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Characterization and Transplantation of Felid Spermatogonial Stem Cells

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Characterization and Transplantation of Felid Spermatogonial Stem Cells

A Dissertation

Submitted to the Graduate Faculty of the
University of New Orleans
in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy
in
Conservation Biology

By

Robin Henry Powell

B.S. Louisiana State University, 2002

May 2015

DEDICATION

To my parents, Wayne and Connie Henry,
who have always provided for me, encouraged me, and
molded me into the person I am today.

To my husband, Chris, who has been
patient and supportive through the final stages of this endeavor.

Finally, to my children, especially Julia,
who had to share so much of my attention with my graduate studies.

In memory of
Stanley P. Leibo,
an exceptional mentor and brilliant mind lost,
and
Monica N. Biancardi,
a bright, talented, unique young woman and friend gone too soon.

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ABSTRACT

Spermatogonial stem cells (SSC) self-renew and differentiate into spermatozoa throughout the life of the male. SSC transplantation is a potential method for the propagation of genetically important males. These cells have been isolated in different mammalian species using specific cell surface markers, but not in felines.

The goal of this study was to explore a relevant strategy for conservation of endangered felids by characterizing domestic cat (*Felis catus*) SSCs and assessing their ability for self-renewal after transplantation. Firstly, SSC and pluripotent surface markers, identified in non-feline species, were tested for expression in mixed germ cells from adults by immunocytochemistry and flow cytometry, with immunohistochemical confirmation of expression in prepubertal and adult testis tissue. Secondly, subpopulations were purified through fluorescence-activated cell sorting using spermatogonia-specific markers and molecularly characterized to ascertain levels of pluripotent transcription factors expressed in cat embryos. Thirdly, subpopulations of mixed germ cells and purified spermatogonial cells were transplanted to prepubertal cats to determine: 1) if SSCs capable of colonization were present, and 2) the value of using adolescent domestic cats without depletion of endogenous germ cells as recipients. Fourthly, various culture conditions were evaluated to identify proteins and factors required to maintain proliferation of cat SSCs. Lastly, adult lion testis tissue was characterized with the same surface markers, and mixed germ cells were transplanted to cat testes to evaluate the cat as a suitable host for lion SSC colonization and differentiation.

Pluripotent surface markers were more reliable than the common SSC surface markers for isolating cat SSCs. Varying expression levels of pluripotent transcription factors between the different purified cell populations identified spermatogonial subpopulations. Cell purification was not necessary to colonize recipient testes, and transplantation validated the use of prepubertal males as recipients without depletion of endogenous cells. Unlike spermatogonia within mixed germ cells, purified spermatogonia were not maintained under various culture conditions; therefore, SSC culture conditions must be optimized. Similarities in the expression patterns of surface markers in lion and cat spermatogonia were revealed, and

colonization of lion SSCs in cat testes provided further evidence of the domestic cat's relevance as a model for exotic felid SSC transplantation.

Keywords: feline, cat, spermatogonia, SSC, SSEA-4, SSEA-1, TRA-1-60, TRA-1-81, GFRA1, SSCT

GENERAL INTRODUCTION

The ART of Felid Conservation

Most of the 36 species of wild felids are listed as threatened or endangered, and captive breeding programs have had low success rates [1]. Death or infertility of a male equates to the loss of his contribution to the genetic diversity of the species and, therefore, a detriment to conservation efforts. Sperm collection by using different assisted reproduction techniques (ARTs) have become an important part of conservation efforts. Although mature spermatozoa can sometimes be collected from a deceased fertile male, the quantity and quality of available sperm and the window for collection post-mortem is limited. Also, if the male was sexually immature or was a seasonal breeder that died outside of breeding season when sperm production may have been suppressed [2], few sperm can be collected.

To overcome some of the above mentioned limitations, different approaches have been explored for post-mortem sperm production. Some studies have reported the successful production of sperm after testis tissue xenografting, a method of *in vivo* culture in which pieces of testis tissue from a donor male are surgically imbedded under the skin of a recipient, usually an immunodeficient mouse [1-4]. This procedure can be applied to immature or mature testis tissue, but there has been little success in exotic species [5, 6]. Additionally, xenografting has several disadvantages, including a limited amount of tissue for grafts, short life spans of recipient mice, possible donor species and age dependent effects determining tissue survival, and intracytoplasmic sperm injection (ICSI) into oocytes is required since sperm have not completed maturation in the epididymis [1, 5, 7, 8].

Spermatogonial stem cell transplantation (SSCT) is another technique that examines *in vivo* sperm production and can circumvent some of the disadvantages observed with testis tissue xenografting. SSCT requires isolating spermatogonial stem cells, a small population of undifferentiated early stage sperm cells, from the testes of the deceased or infertile male and transplanting the cells into the testes of an appropriate living recipient. An advantage to this technique is that the stem cell-like spermatogonia differentiate into spermatozoa while concomitantly replenishing themselves through mitotic division [9]. The maintenance of

proliferating spermatogonia in an *in vitro* culture system could provide a potentially unlimited supply of these valuable cells [9] and allow for transplantation to multiple recipients.

While advances in current ART can be a significant asset to the conservation of felids, other techniques are being sought to further aid those efforts [10-12]. Inter-species SSCT may substantially benefit felid conservation efforts, as shown by successful ocelot SSCT to domestic cat testes [13]. In the present study, I investigated the domestic cat (*Felis catus*) as a model for optimizing SSCT for future application to other felids.

The Spermatogonial Stem Cell Environment:

Anatomy and Function of the Testis

In addition to producing some of the male hormones, the testis functions to generate gametes in the form of spermatozoa to allow the passage of genetic information to his offspring. This reproductive organ is primarily composed of numerous lobules of long, coiled tubules called seminiferous tubules that provide the complex environment in which spermatozoa are formed through a highly coordinated process called spermatogenesis. Spermatogenesis begins as a male approaches sexual maturity, as early as five months of age in the domestic cat [14], and is largely regulated by the Sertoli cell, a somatic cell found within the seminiferous tubule. Among several other functions, Sertoli cells provide many of the factors and nutrients vital for germ cell development and aid in germ cell movement as they progress through spermatogenesis [15]. The precursors of spermatozoa are single diploid cells called spermatogonia that occupy the basal compartment, a microenvironment formed by the basement membrane of the tubules and the supporting Sertoli cells that extend into the adluminal compartment of the seminiferous tubule. Adjacent Sertoli cells form junctional barriers, separating the spermatogonial cells in the basal compartment from the other stages of germ cells in the adluminal compartment [15].

Spermatogonia are divided into subclasses, and in most mammals, the earliest spermatogonial cells are a small population of germ cells termed type A single (A_s) spermatogonia present from birth [16, 17]. These are undifferentiated cells capable of both self-renewal and producing daughter cells for differentiation and are thus considered spermatogonial stem cells (SSCs). During spermatogenesis, the A_s spermatogonia replicate to

form pairs (A_{pr}) or chains, or aligned (A_{al}), spermatogonia connected by intercellular bridges, most of which then undergo apoptosis or are fated to differentiate [18-20]. Some A_{al} spermatogonia differentiate and undergo several mitotic divisions (A_{1-4}) to develop into intermediate (In) spermatogonia, and subsequently type B spermatogonia. As spermatogenesis progresses into the adluminal compartment, spermatocytes formed from mitotic division of type B spermatogonia undergo two meiotic divisions to produce haploid spermatids. The round spermatids are structurally modified to yield the spermatozoa with flagellum extending into the lumen. Unlike SSCs, spermatozoa are not able to replicate, and therefore cannot be expanded *in vitro* to increase the number of available gametes.

Spermatogonial Stem Cell Transplantation:

An Overview

Brinster and Zimmerman first introduced the technique of SSCT in 1994 by restoring spermatogenesis in infertile mice with the transplantation of mixed germ cells from fertile mice [21]. Spermatogonia isolation has since been reported in fish and several mammalian species, including various rodents, livestock, non-human primates, and humans, with successful spermatogenesis in some species [22-26]. Briefly, SSCs are isolated from a donor testis and transplanted to the testes of an appropriate male recipient. However, these two main aspects of the procedure involve several important considerations: 1) how the donor cells are obtained, 2) the species of the recipient, 3) how or if the recipient's endogenous cells will be depleted, 4) the method of donor cell transplantation to the recipient, and 5) how the donor cells will be identified if donor sperm are produced. Each of these factors impacts the efficacy of SSCT and also presents some constraints.

Isolating donor cells. Spermatogonial stem cells are few in number in the testis, comprising only 0.02 – 0.2% of the testicular cells in adult rodents [27, 28], and although transplantation of mixed germ cells obtained by enzymatic digestion has been successful [21, 29], enrichment for SSCs may allow for increased colonization efficiency. Several studies have reported using cell populations enriched for SSCs by one or more purification methods, including differential plating [24, 28], density gradient separation [13, 28], and cell sorting using cell surface markers expressed by SSCs [20, 30-32]. Identification of SSC-specific surface

markers has gained the most focus and also offers other advantages such as a better understanding of the SSC biology. Surface markers will be further discussed later.

Selecting a SSCT recipient. Autologous (same self), syngenic, (same species), and xenogenic (across species) SSCT have been attempted with some success. For SSCT resulting in the production of donor sperm, it is essential to select a recipient that can provide a biologically compatible environment for the donor cells. As phylogenetic distance between donor and recipient species increases, the biological differences may become too great for donor cell spermatogenesis to occur [9, 33, 34]. Most early studies used mice as recipients, regardless of donor species, to show that donor cells were SSCs capable of colonization [9, 33-35]. However, transplantation of cells from non-murine species, with the exception of some other rodents, was unsuccessful in establishing spermatogenesis despite population of the seminiferous tubules [9, 28, 33, 36]. More recent studies investigating syngenic transplantation in other species, including canine [22, 29], porcine [37, 38] and bovine [23], and a few xenogenic transplantations with phylogenetically related, non-rodent species have been successful [13, 39, 40]. One advantage of xenogenic SSCT is the ability to collect the sperm of an exotic species donor from a compatible domestic species recipient that is generally more manageable.

Preparing recipients for SSCT. Conventionally, recipients are treated prior to SSCT to eradicate endogenous cells, reducing competition and providing an open niche for donor cells to colonize. Additionally, this helps prevent the contamination of potential donor sperm by recipient sperm. The most common method to deplete the endogenous cells in small animals is with busulfan injection [9, 17, 33, 34], a cytotoxin commonly used to destroy cancer cells, while focal irradiation of the testes is frequently used in larger mammals [9, 13, 23, 29]. Other alternatives include induced cryptorchidism [17] and the use of sexually immature males with fewer endogenous cells present [27, 38, 39, 41].

Transplanting SSCs. The species of the recipient influences the method of injection as testicular size and structure may vary. In initial studies, direct injection into a seminiferous tubule was done [21], but other sites of injection including the efferent ducts, which connect the head of the epididymis to the testis and the rete testis, were later explored [28, 36]. In some studies, ultrasound guidance has been used to inject through the tail of the epididymis

into the rete testis, particularly in larger mammalian species [23, 29, 38, 39]. In the cat, injection through an external portion of the rete testis has been sufficient [13].

Confirming donor cell colonization. A method to distinguish donor cells from recipient cells may be required to confirm successful SSCT since contamination with endogenous cells can occur if ablation is not 100% or natural re-establishment of spermatogenesis occurs after depletion [13, 23]. Regardless of donor and host species, the origin of germ cell stages prior to spermiogenesis would be difficult to distinguish in xenogenic transplantation. Also, sperm in related species may appear morphologically similar. One of the most common techniques for distinguishing donor cells from the recipient's cells is viral transfection of Green Fluorescent Protein (GFP), where either a transgenic host is used or the donor cells themselves are transfected [28, 42]. A temporary solution is fluorescent labeling of donor cells with PKH26, a red lipophilic membrane dye stable up to 100 days or 10 generations *in vivo* [13, 24, 28, 39].

Phenotypic and Molecular Characterization of Spermatogonial Stem Cells

Phenotypic characterization. Specific cell types have their own unique combination of proteins on their cellular surface, helping to identify them as that particular type of cell. These surface markers have been used in several mammalian species to phenotypically characterize SSCs and, therefore, for further purification by fluorescence activated cell sorting (FACS) or magnetic activated cell sorting (MACS). Frequently used markers include **KIT, CD9, ITGA6, GFRA1, GPR125, and THY1**. In more recent studies, the use of pluripotent surface markers such as **SSEA-1, SSEA-4, TRA-1-60, and TRA-1-81** has been investigated due to the stem cell-like ability of SSCs to self-renew. However, the pattern of expression of these markers can vary by species, and they have not yet been fully explored in the cat (Table i).

KIT (CD117), a proto-oncogene and a member of the tyrosine kinase family, is a marker of differentiating germ cells [18, 30, 43]. Kit was only detected in differentiating spermatogonia and in early preleptotene spermatocytes in mice [43]. In humans, KIT was found to be expressed only in later germ cells, including spermatocytes and round spermatids [30]. Therefore, KIT may be used as a marker to separate differentiating germ cells from undifferentiated spermatogonia in the cat, as was done in mice [44].

CD9, a member of the transmembrane 4 superfamily known as tetraspanins, is involved in cell adhesion, differentiation, migration, and proliferation. CD9 is expressed on several types of stem cells, including SSCs in the mouse [31, 45, 46] and rat [45]. However, there was also concurrent expression of CD9 and Kit in some cells, suggesting CD9 is also expressed in differentiating spermatogonia in the mouse and rat [45]. Another study showed CD9 positive cells were more often in paired or chains of spermatogonia [46]. Cells positive for CD9 have been separated from other germ cells using MACS [45, 46].

Integrin alpha-6 (ITGA6 or CD49f), an integral cell surface protein that participates in cell adhesion and cell signaling, can also be expressed by stem cells [31]. In mouse SSCs, Itga6+ cells were found in cell populations that were also positive for expression of CD9 and Gfra1 [31]. In humans, ITGA6 is not only expressed in spermatogonia, but also in Sertoli cells and Leydig cells [30]. Therefore, it may be important to remove any somatic cells by differential plating after sorting with ITGA6.

Glial cell line-derived neurotrophic factor family receptor alpha 1 (GFRA1) is part of the receptor complex for glial cell line-derived neurotrophic factor (GDNF), which is secreted by Sertoli cells to regulate the proliferation and self-renewal of SSCs [47, 48]. Sorting for Gfra1 positive cells in mouse testes using MACS provided enriched SSC populations and showed that subpopulations of SSCs expressing higher levels of Gfra1 also expressed the pluripotent marker *Pou5f1* (*Oct4*) [31]. In a recent study, it was shown that GFRA1 expression was also specific to spermatogonia in humans [30]; however, in a previous study, expression of GFRA1 in Sertoli cells and Leydig cells was found [49].

G protein-coupled receptor 125 (GPR125), an orphan adhesion-type G-protein-coupled receptor, has been found to be expressed exclusively in undifferentiated spermatogonia in the mouse [44, 48]. A subpopulation of undifferentiated spermatogonia in the human was found to be positive for GPR125 as demonstrated by only one or two positive cells per seminiferous tubule cross-section [30]. Human undifferentiated spermatogonia positive for GPR125 have been selected using MACS [30].

Glycosyl phosphatidylinositol-anchored surface antigen (THY1 or CD90) is a cell surface antigen that has been used as a marker for undifferentiated spermatogonia in adult testes [18]. In one study, mouse germ cells positive for Gfra1, CD9, or Thy1 sorted using MACS were found to be separated into cell populations of the same size and granularity pattern [46]. THY1 positive cells were found in a subpopulation of human spermatogonia and co-expressed with ITGA6, GFRA1 and GPR125 [30]. If many of these surface markers co-express, then it will only be necessary to use one or two for cell sorting.

The stage-specific embryonic antigens SSEA-1 and SSEA-4 vary in their expression pattern among species [50, 51]. Mouse embryonic stem (ES) cells and embryonic germ (EG) cells express SSEA-1, but neither express SSEA-4 [50]. Human ES cells express SSEA-4, but not SSEA-1 [50-53]. Human EG cells, however, express both SSEA-1 and SSEA-4 [50, 51]. In one study, ES-like cells expressing SSEA-4 were derived from testicular cells of adult men [54]. Cat ES cells were found to express SSEA-1, but not SSEA-4 [55]. Since these markers are expressed in stem cells and embryonic germ cells, they may be useful in identifying the stem cell-like germ cells in cat testes.

Two other widely used stem cell surface markers tumor rejection antigens TRA-1-60 and TRA-1-81 are often found co-expressed with SSEA-4 on stem cells in humans and nonhuman primates, and that expression, as with other pluripotent markers, is lost as cells differentiate [51, 53, 56, 57]. These markers have also been detected in embryonal carcinoma cells and germ cell tumors [58]. Their molecular identities have long remained a mystery, but recent studies have found that these antigens recognize different epitopes on the same large glycoprotein [56-59].

Molecular characterization. Similar to embryonic stem cells (ESCs), SSCs can exhibit pluripotent transcription factor expression, which is usually downregulated as either of these cell types begins differentiating. Domestic cat ESCs expressed the pluripotent transcription factors **NANOG**, **POU5F1**, and **SOX2** [55, 60], and Verma et al. [61] reported that insertion of those genes into snow leopard (*Panthera uncia*) fibroblasts generated induced pluripotent stem cells. In human ESCs, **NANOG** and **POU5F1** were shown to co-express with surface markers

SSEA-4, TRA-1-60, and TRA-1-81 in various combinations, with some ESCs co-expressing all five markers [53].

More importantly, expression of *NANOG*, *POU5F1*, and *SOX2* was also discovered in human and mouse primordial germ cells (PGCs), the precursors to oogonia and spermatogonia [62-64], and their expression in testicular cells has been investigated in several species. *NANOG* was detected in differentiating germ cells of human, porcine, dog, and mouse testis, and weak expression was observed in type A spermatogonia [65]. Conversely to *Nanog*, *Pou5f1* expression is downregulated in replicating spermatogonia committed to differentiation in mice [64] and in a subpopulation of human germ cells isolated from fetal testis at the second trimester of gestation [66], but expressed in undifferentiated SSCs with higher levels of expression in SSEA-4+ spermatogonia from adult human testis compared to the levels observed in the whole testis [32]. Moreover, a recent study demonstrated the importance of *POU5F1* for colonization activity after transplantation [67]. In fact, transplantation of mice testicular cells marked with EGFP+ at the *Pou5f1* locus resulted in a 30-fold increase in colonization compared to that of EGFP- cells [67]. The biological function of *SOX2* in SSCs seems to differ from that of self-renewal by *POU5F1*. *SOX2* is not expressed by human PGCs [63], but its expression is required for PGC proliferation in mice [62], possibly for regulation of Kit, which is essential for germ cell proliferation [62]. Given these findings, it is plausible that pluripotent transcription factors may play an important role in the stem cell activity of SSCs, and expression of these factors in cell subpopulations obtained by cell sorting with surface markers may help determine the efficacy of those surface markers for SSC enrichment.

Table i. Marker expression varies among the mouse, human, and cat, with expression of most markers not yet evaluated in cat testicular cells.

Marker		Cell Type by Species		
Marker Type		Mouse [31, 43-46, 48, 50, 62, 64, 65]	Human [30, 49-54, 57-59, 63, 65, 66]	Cat [13, 55, 60, 68, 69]
SSC Surface Markers	KIT	Dif s'gonia, s'cyte	S'cyte, s'tid	X
	CD9	SSC dif s'gonia	S'gonia	?
	ITGA6	SSC	S'gonia, Sertoli, Leydig	?
	GFRA1	SSC	S'gonia, Sertoli, Leydig	S'gonia
	GPR125	SSC	SSC	?
	THY1	S'gonia	S'gonis	?
Pluripotent Surface Markers	SSEA-1	ES, EG, SSC	EG	ES
	SSEA-4	X	ES, EG, SSC	Dif ES
	TRA-1-60	X	ES, EG	?
	TRA-1-81	X	ES, EG	?
Pluripotent Transcription Factors	NANOG	PG, s'gonia, s'cyte, s'tid	ES, PG, Dif germ cells	ES
	POU5F1	PG	ES, PG	ES
	SOX2	PG	ES	ES

Dif, differentiating; S'gonia, spermatogonia; S'cyte, spermatocyte; S'tid, spermatotid; SSC; spermatogonial stem cell; ES, embryonic stem cell; EG, embryonic germ cell; PG, primordial germ cell.

Spermatogonial Stem Cells in Culture

Culturing of SSCs allows for the study of their biology to exploit their ability to self-renew or differentiate for future uses. In several studies, the maintenance and proliferation of SSCs in culture for unlimited supply, providing cells for multiple SSCTs and other ARTs have been investigated [29, 30, 33, 70]. Spermatogonial stem cells are few in number in the testis [27, 28], and during *in vitro* culture, they may be crowded out by faster replicating somatic cells. Therefore, it is necessary to develop methods to eliminate somatic cells and germ cells in other stages of spermatogenesis to prevent contamination of the SSC cultures.

It is important to formulate the right conditions in which to culture SSCs. The SSCs need to be maintained in an undifferentiated state while also promoting proliferation, allowing for

their continuous availability for SSCT. Without the proper signaling factors, SSCs will begin to differentiate. In the seminiferous tubules, the undifferentiated spermatogonia are located between the basement membrane and the Sertoli cells, and signals from the Sertoli cells regulate the state of the spermatogonia [15, 18]. GDNF, secreted by the Sertoli cells *in vivo*, has been a commonly used factor in SSC culture medium [30, 32, 71, 72], and an overexpression of GDNF induces an accumulation of undifferentiated spermatogonia in mice [47].

Progress in Feline Spermatogonial Stem Cell Research

Xenografting of feline testis tissue. Xenografting of cat tissue was first performed in 2004 by Snedaker et al. [1] using fresh testis tissue from one- to five-week-old kittens, and following grafting under the skin of male immunodeficient mice, resulting in full spermatogenesis at 36 weeks post transplantation. However, Kim et al. [8] found that donor tissue survival was age-dependent, resulting in sperm production in tissue from sexually immature cats and degeneration of tissue from donors that reached puberty (≥ 8 mo). The first attempt of xenografting wild felid testis tissue was recently reported using Iberian lynx (*Lynx pardinus*) testis tissue samples from donors of various ages (6-week-old fetus to 2-year-old sub-adult) [5]. Most of the tissue samples used in this study were cryopreserved, and spermatogonia were observed in 10-15% of seminiferous tubules only in donor tissue from 6-month-olds while the tissue from all other lynx donors appeared to have degenerated [5]. These results highlight some disadvantages with xenografting, including the effects of donor species and age and possible effects of tissue storage methods, which may be overcome using SSCT as an alternative technique for *in vivo* culture.

Isolation and transplantation of feline SSCs. Domestic cat mixed germ cells have been isolated by enzymatic digestion of testis tissue [9, 13, 73] and transplanted into the testes of immunocompetent mice [9] and adult domestic cats [13] in which endogenous germ cell depletion was performed by either chemical or radiation treatment. Although cat SSCs were able to colonize testes of both species, only donor cells transplanted to cat recipients were able to differentiate into sperm, supporting the suggestion that phylogenetic distance plays a role in donor-recipient compatibility [9, 13]. Silva et al. [13] also reported the successful xenogenic transplantation of ocelot mixed germ cells, resulting in donor-derived sperm production, which

indicates that the domestic cat may be a suitable recipient for xenogenic transplantation of other felid SSCs.

Characterization of feline SSCs. Immunohistochemical staining of cat testis fragments and purified spermatogonia obtained by centrifugation over a Percoll density gradient and subsequent differential plating revealed cat spermatogonia were positive for the SSC surface marker GFRA1 [13]. Juvenile and adult cat testis fragments and enzymatically isolated spermatogonia, purified by differential plating, were molecularly analyzed to evaluate the expression of several SSC markers, including surface markers *GPR125*, *GFRA1*, and *THY1*, and intracellular marker *POU5F1* [68]. RT-PCR analysis detected mRNA for all four markers in both whole testis and single-cell suspensions from the juvenile and adult tissue, suggesting that these common SSC markers may be conserved among species [68] and confirming that these surface markers are good candidates for isolating cat SSCs.

***In vitro* culture of cat SSCs.** Tiptanavattana et al. [73] reported the first study of long-term maintenance of cat SSCs *in vitro*. In addition to a recombinant rat GDNF, the growth medium was supplemented with other factors commonly used in stem cell cultures, including mouse epidermal growth factor (EGF), human basic fibroblast growth factor (bFGF), and recombinant human leukemia inhibitory factor (LIF). Initially, floating SSCs were negatively selected for after testicular somatic cells attached to the dish. These SSCs were passaged to new dishes, and proliferating colonies of 3-6 cells were observed by day 13-15 of culture. These colonies were then plated on feeder layers of mouse embryonic fibroblasts or cat Sertoli cells where they continued proliferating until colony degeneration was observed on day 57. Long-term culture beyond that was not achieved, indicating *in vitro* culture conditions for cat SSCs still need to be optimized as more is understood about these cells.

Introduction to the Following Chapters

In the following chapters, I explore the key facets of SSCT in relation to the domestic cat. The cat has been utilized as a model for other ARTs in felids [10-12], and SSCT may be a relevant tool for conservation of endangered felids. It has already been demonstrated that, in addition to successful SSCT with domestic cat mixed germ cells, ocelot mixed germ cells were able to colonize the testes of domestic cats and progress through spermatogenesis without SSC

enrichment [13]. However, it has been proposed that cell populations enriched for SSCs may improve colonization, and SSCs in several species including mice and humans have already been characterized for selection [20, 45, 48, 74]. There has been little investigation into the biology of feline SSCs, and markers identified in domestic cat SSCs may also aid in isolation of SSCs for conservation of other felids. Therefore, the goal of this dissertation is to characterize and isolate SSCs for transplantation in the domestic cat in anticipation of applying this model to other felid species.

Chapter 1. Phenotypic and molecular characterization of domestic cat (*Felis catus*) spermatogonial stem cells. The major challenge for isolation and purification of SSCs is definitive identification of specific SSC markers. Surface markers have been used to enrich SSCs in several mammalian species, however their expression varies by species, and cat SSCs have not been fully characterized. In this chapter, I assessed the expression of several SSC surface markers commonly used in other species in addition to some pluripotent surface markers to identify markers for isolating cat SSCs. Mixed germ cell suspensions were stained with each marker and positive cells were quantified by flow cytometry. The types or stages of cells expressing each marker were determined by immunohistochemistry in testis tissue. Lastly, subpopulations expressing markers assumed to identify cat SSCs were isolated by FACS and evaluated by qRT-PCR for the expression levels of pluripotent transcription factors. As stem cell-like cells, SSCs express some pluripotent markers also expressed by ESCs.

Chapter 2. Successful Colonization of SSEA-1+ and SSEA-4+ Domestic Cat Spermatogonial Subpopulations after Transplantation into Prepubertal Males without Depletion of Endogenous Germ Cells. Transplantation of SSCs may be an effective method to propagate genetically important males. The two main features of SSC transplantation are isolation of donor SSCs and selection of a proper recipient with an available niche for donor cells to colonize. Although the overall technique is similar for all species, the exact methods may be species-specific. In this chapter, I assessed the efficacy of transplanting cell populations enriched for cat SSCs and the need for endogenous germ cell depletion in recipients. To address the former, the colonization ability of mixed germ cell populations was compared to that of subpopulations sorted for SSEA-1 or SSEA-4. In an effort to avoid the need for chemical

or radiation treatments, the recipients were five- and six-month-old cats in which spermatogenesis either had not started or was just beginning. If an available niche is present in adolescent recipients, donor cell colonization should be detected, and if SSC enrichment improves efficiency, greater colonization of SSEA-1 and SSEA-4 positive cells should be observed as compared to that seen after transplantation of mixed germ cell.

Chapter 3. Developing a Domestic Cat Spermatogonial Stem Cell Culture. Maintenance of proliferating SSCs *in vitro* would not only provide a better understanding of cat SSC biology, but also allow expansion and production of a continuous supply of SSCs to use for assisted reproduction techniques. Although SSC culture methods have been largely based on those used to culture stem cells in other species, the SSC environment is complex and requires specific factors and nutrients which may vary by species. In this chapter, the effects of the presence of a feeder layer, in addition to various factors and protein supplements, on mixed germ cell populations and sorted cell subpopulations were evaluated. First, mixed germ cells were cultured in medium either with or without FBS to evaluate the necessity for serum protein supplement. Next, sorted cells were cultured either on a feeder layer of Sertoli cells or on a plastic surface to determine if a feeder layer benefits SSC growth *in vitro*. Finally, various combinations of growth factors were added to cultures of sorted cells to identify those that may promote SSC proliferation. The optimal cell culture condition should allow long-term maintenance of cat SSCs.

Chapter 4. Phenotypic Characterization and Xenogenic Transplantation of Lion Spermatogonial Cells. The ability to obtain wild felid sperm after SSCT to a domestic recipient would be a valuable achievement for felid conservation. Xenogenic transplantation of ocelot mixed germ cells to the domestic cat has been successful [13]; however, the phylogenetic distance from other felid species may affect the compatibility of the domestic cat as a recipient. Identifying biological similarities, such as SSC surface markers, between the cells and testicular environments of the domestic cat and other felids may be an indication of SSCT compatibility. In this chapter, pluripotent surface markers and SSC surface markers were evaluated in lion testis for comparison to surface marker expression in the cat; then, lion mixed germ cells were transplanted into testes of prepubertal domestic cats. If the lion and cat SSCs share a similar

expression pattern, then the domestic cat may be a good candidate as a recipient for lion SSCs, which could be confirmed by colonization of lion SSCs and sperm production following transplantation.

CHAPTER 1

PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF DOMESTIC CAT (*Felis catus*) SPERMATOGONIAL STEM CELLS

Abstract

In many mammalian species, surface markers have been used to obtain enriched populations of spermatogonial stem cells (SSCs) for assisted reproduction and other applications; however, little is known about the expression patterns of feline SSCs. In this study, we assessed the expression of SSC surface markers commonly used in other species – KIT, ITGA6, CD9, GFRalpha1, GPR125, and THY1 – in addition to the less frequently used pluripotent markers –TRA-1-60, TRA-1-81, SSEA-1, and SSEA-4 – in SSCs of both prepubertal and adult domestic cats (*Felis catus*). To further characterize cat SSCs, we sorted cells using SSC-specific markers and evaluated the expression of pluripotent transcription factors *NANOG*, *POU5F1*, and *SOX2* and the proto-oncogene *MYC* within these populations. We concluded that SSC surface markers used in other mammalian species were not specific for identifying cat SSCs. However, the pluripotent markers we evaluated were more specific to cat spermatogonia, and the presence of SSEA-1 and SSEA-4 in fewer and primarily individual cells suggests that these two markers may be used for enrichment of cat SSCs. The expression of pluripotent transcription factors at mRNA level by single-stained cells positive for SSEA-4 and dual-stained cells positive for both GFRalpha1 and SSEA-4 reflects the undifferentiated stage of cat SSCs, while the absence of transcription factors in double stained cells imply the loss of the stem cell-like identity with the loss of either GFRalpha1 or SSEA-4. Further investigation is warranted to elucidate the biological characteristics of these spermatogonial subpopulations.

Introduction

Spermatogonial stem cells (SSCs), progenitor cells capable of both self-renewal and differentiation into spermatozoa, have been successfully isolated from the testes of several mammalian species. Following transplantation to a proper host for differentiation, SSCs have

the potential to allow genetically important males to propagate – a promising strategy for preservation of endangered species. For example, recently in felids, ocelot mixed germ cells were transplanted successfully to domestic cat testes [13].

SSCs comprise only a small percentage of germ cells occupying the seminiferous tubules [27, 28], and using surface markers for SSC enrichment may improve such applications as transplantation and culture and provide a better understanding of germ cell biology. In numerous studies, the expression of specific cell surface markers has been described, particularly in mouse and human testicular cells, allowing the identification and purification of SSCs. In fact, surface marker proto-oncogene KIT (C-Kit or CD117) has been used across species as an identifier for differentiating spermatogonia and subsequent germ cell stages [18, 30, 43, 44]. Membrane surface markers, such as ITGA6 (integrin alpha-6 or CD49f), CD9 (CLB-thromb/8), glial cell line-derived neurotrophic factor family receptor alpha 1 (GFRA1), G protein-coupled receptor 125 (GPR125), and THY1 (CD90) have been also used for identifying and isolating spermatogonial cells in the mouse [31, 44-46, 48], monkey [75], and human [18, 30, 49]. Additionally, some of these membrane surface markers can be expressed in differentiating or somatic cells, depending on the species [20, 30, 32, 45, 49, 76, 77]. To date, GFRA1 is the only surface marker shown to be expressed by cat SSCs and used for germ cell purification [13, 73]. Nonetheless, GFRA1 may not be an adequate marker to identify and purify cat SSCs. In preliminary studies, we found that GFRA1 is also expressed by differentiated cat germ cells [78, 79]. Clearly, the expression of SSC specific markers in felids has not been fully characterized.

Although SSCs are stem cell-like, pluripotent markers have been used infrequently for SSC identification or purification. The stage-specific embryonic antigens 1 and 4 (SSEA-1 and SSEA-4) and tumor-rejection antigens TRA-1-60 and TRA-1-81 are glycan stem cell surface antigens expressed in different combinations depending on the lineage of pluripotent cell, the state of differentiation, and the species [51, 52, 80]. In cat embryonic stem (ES) cells, undifferentiated colonies expressed SSEA-1 while SSEA-4 was detected in populations starting to differentiate [55]. These markers have not been evaluated in cat SSCs, but one or more have

been identified in the SSCs of mice [81], humans [20, 32, 82], and different species of monkeys [74, 80].

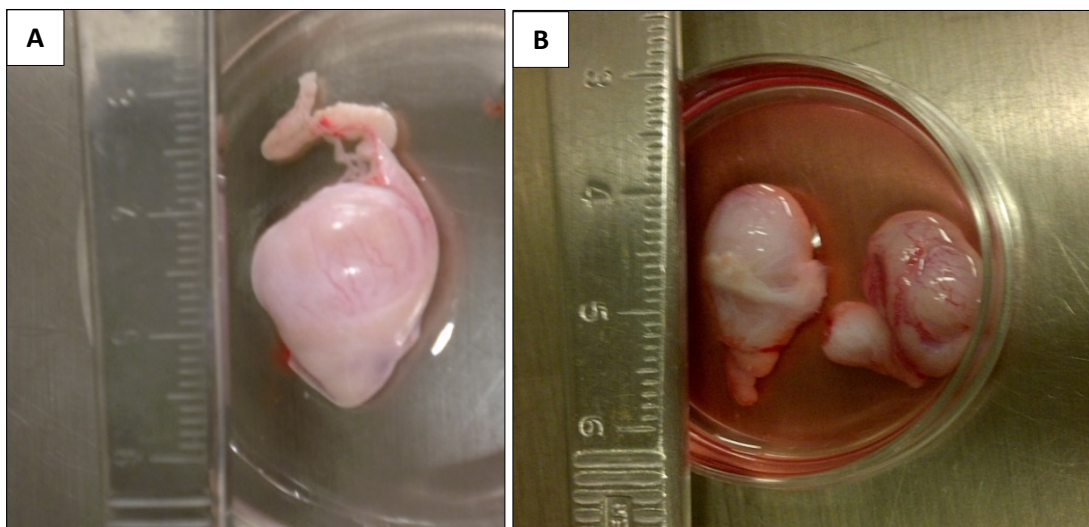
The purpose of the present study was to identify surface markers specific to SSCs in the domestic cat as a model for wild felid conservation. Specifically, we assessed whether (1) prepubertal and adult domestic cat SSCs express surface markers commonly used in other species, in addition to pluripotent markers frequently used in embryonic stem cells and, (2) sorted cat SSCs express pluripotent transcription factors *NANOG*, *POU5F1*, and *SOX2*.

Materials and Methods

Chemicals. All chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

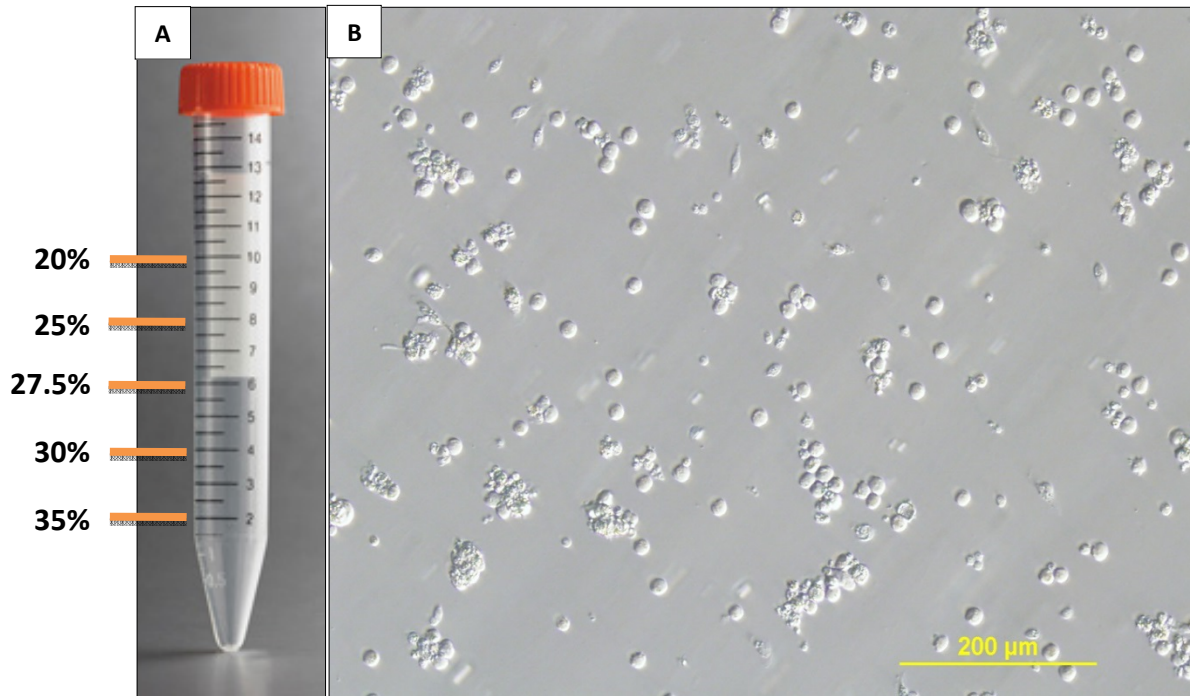
Tissue procurement and handling. Testes from prepubertal and sexually mature domestic cats, as determined by weight [14] and general size (Fig 1.1), were obtained from local veterinary clinics after routine castration. Tissue was transported in HEPES buffered saline (Ultrasaline A Solution, Bio-Whittaker, Walkersville, MD, USA) and either processed immediately or stored \leq two days at 4°C. Prior to use, testes were rinsed in Hanks' Balanced Salt Solution (HBSS). Tunica vaginalis and epididymal tissue were carefully removed so as to minimize damage to the tunica albuginea and underlying seminiferous tubules. Trimmed testes were rinsed in fresh HBSS and weighed before fixation or tissue isolation.

Figure 1.1. Domestic cat testis size increases with spermatogenic function. Adult (A), early pubescent (B, left), and prepubertal (B, right) testes.



Spermatogonial stem cell isolation. After preparation as described above, the tunica albuginea were gently dissected away from the seminiferous tubules of prepubertal and adult testes, and tubule tissue was incubated for 30-45 min in HBSS containing 10 µg/mL amphotericin-B, 10 µg/mL streptomycin, and 50 µg/mL gentamicin, and then rinsed and minced in fresh HBSS. Mixed testicular cell suspensions were obtained using a two-step enzymatic digestion following the protocol described by Kim et al. [9], with minor modifications, and followed by filtration and density gradient separation as described below. Minced tubule tissue was incubated in 4 mL HBSS containing 0.5 mg/mL collagenase type IA and 0.5 mg/mL collagenase type IV (Life Technologies/Fisher, Grand Island, NY, USA) per testis at 34°C in a shaking water bath set at 150 oscillations/min for 20 min or until dissociation into individual tubules was achieved. Dispersed seminiferous tubules were allowed to settle for 5 min in HBSS on a cold block, and supernatant was siphoned by pipette. Tubules were rinsed 3X with fresh HBSS, allowing the tubules to settle and removing the supernatant between each rinse. The final supernatant was removed and the tubules were digested further with 2 mL trypsin (0.25%; Cellgro, Manassas, VA, USA) and 1 mL DNase I (3 mg/mL) per testis at 34°C in a shaking water bath set at 150 oscillations/min for 20 min or until a single cell suspension was obtained. An equal volume of DMEM/F12- (Gibco, Frederick, MD, USA) supplemented with 15% Knockout Serum Replacement (Invitrogen, Carlsbad, CA, USA), 5% FBS (Hyclone, Thermo Fisher Scientific, Waltham, MA, USA), 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, and 1.25% non-essential amino acids (SSC medium) was added, and the cell suspension was filtered through 100 µm and 40 µm nylon mesh (Corning, Corning, NY, USA). Cell suspensions were centrifuged at 725 x g, for 25 min over a 5-layer density gradient (35.0%, 30.0%, 27.5%, 25.0%, 20.0%; 1.5 mL/layer; Fig 1.2A) of Percoll (GE Healthcare Life Sciences, Piscataway, NJ, USA) in a 15mL tube. Cell suspensions visualized at the interfaces between the 35.0% to 30.0%, 30.0% to 27.5%, and the 27.5% to 25.0% layers were pooled (Fig 1.2B), rinsed with Dulbecco's phosphate buffered saline (DPBS), and pelleted by centrifugation for 5 min at 220 x g for three washes. Pellets from adult testes were used for ICC, FC, or fluorescence activated cell sorting (FACS).

Figure 1.2. Cells were isolated using a Percoll density gradient (A). Population of mixed germ cells isolated from the bands formed at the 30.0%, 27.5%, and 25.0% Percoll layers (B).



Immunocytochemistry (ICC). The list of antibodies used for ICC is described in Table 1.1. Pellets of mixed germ cells, purified by density gradients of Percoll, were fixed with 4% formaldehyde in DPBS for 20 min at room temperature (rt). After fixation, cells were washed 3X with DPBS and blocked with 5% serum + 1% bovine serum albumin (BSA) in DPBS overnight at 4°C. Cells were then aliquoted into 1.5 mL conical tubes and incubated with primary antibodies: ITGA6, CD9, GFRA1, GPR125, THY1, KIT, SSEA-1, SSEA-4, TRA-1-60, and TRA-1-81 for 1.5 h, followed by 1 h exposure to secondary antibodies: Alexa Fluor 555 goat anti-rabbit IgG for KIT, GFRA1, and GPR125; sheep anti-mouse IgG-FITC conjugate for CD9, THY1, and SSEA-4; and goat anti-mouse IgM CY3 conjugate for SSEA-1, TRA-1-60, and TRA-1-81. Cells were counterstained with DAPI (12.5 ng/mL; Invitrogen/Life Technologies, Carlsbad, CA, USA), and observed using fluorescence microscopy. Controls for nonspecific auto-fluorescence were performed by replacing the primary antibody with IgG serum specific to the host of the

secondary antibody and incubating the cells with the secondary conjugated antibody only. Fluorescence was detected using an Olympus BX60 microscope, and images captured with an Olympus DP72 digital camera.

Flow cytometry (FC) and fluorescence activated cell sorting (FACS). Mixed germ cell suspensions from either a single testis or pooled from multiple testes of adult cats were filtered through 40 μm nylon mesh, rinsed with DPBS, and blocked in 5% serum + 1% BSA in DPBS for 45 min. Cells were then incubated with each of the primary antibodies positively detected by ICC: KIT, GFRA1, GPR125, SSEA-1, SSEA-4, TRA-1-60, and TRA-1-81 for 1.5 h, followed by exposure to secondary antibodies Alexa Fluor 647goat anti-rabbit for KIT, GFRA1, and GPR125; sheep anti-mouse IgG-FITC conjugate for SSEA-4; and Alexa Fluor 488 goat anti-mouse for SSEA-1, TRA-1-60, and TRA-1-81 for 45 min (Table 1.1) on ice while on a rocker platform. Cells stained with single or dual antibodies (GFRA1 and SSEA-4) were re-suspended in 1 mL DMEM/F12 per 10×10^6 cells. Controls for nonspecific binding were performed by replacing the primary antibody with either sheep or goat IgG and secondary antibody only, and unstained cells as control for auto-fluorescence.

To quantify the antibody expression, 10×10^3 to 50×10^3 cells per antibody in three replicates were analyzed using two flow cytometers (FACSCalibur and FACSARIA, Becton Dickinson Biosciences, San Jose, CA, USA). Percentages of cells expressing each antibody were calculated using CellQuest (Becton Dickinson Biosciences, San Jose, CA, USA), FACSDiva (Becton Dickinson Biosciences, San Jose, CA, USA), and FlowJo (Tree Star, Ashland, Oregon, USA) software. Cells (up to 10×10^7) single stained for SSEA-1 or SSEA-4, or double stained for both GFRA1 and SSEA-4 were sorted, and positive cells collected into 5 mL round bottom tubes containing 1mL DMEM/F12, 20% Knockout Serum Replacement, 1 mM L-glutamine, 0.1 mM β -mercaptoethanol, 1.25% non-essential amino acids, 10 μM Y27632 ROCK-inhibitor (Stemgent, Cambridge, MA, USA), 10 $\mu\text{g/mL}$ amphotericin-B, 10 $\mu\text{g/mL}$ streptomycin, and 50 $\mu\text{g/mL}$ gentamicin.

Table 1.1. List of primary and secondary antibodies used for characterization of cat SSCs.

	Host - antibody type	Source	Working Dilution	Method
Primary antibodies				
KIT	Rabbit polyclonal	Abcam (Ab5506)	1:100	ICC, IHC, FC
CD9	Mouse monoclonal	Abcam (Ab51380)	1:100	ICC
ITGA6	Rat monoclonal	Biolegend (313601)	1:50	ICC
GFRA1	Rabbit polyclonal	Abcam (Ab8026)	1:50	ICC, IHC, FC
GPR125	Rabbit polyclonal	Abcam (Ab51705)	1:100	ICC, IHC, FC
THY1	Mouse monoclonal	Santa Cruz (Sc59396)	1:200	ICC
SSEA-1	Mouse monoclonal	Millipore (MAB4301)	1:50	ICC, IHC, FC
SSEA-4	Rat monoclonal	Millipore (MAB4303)	1:50	ICC, IHC, FC
TRA-1-60	Mouse monoclonal	Millipore (MAB4360)	1:50	ICC, IHC, FC
TRA-1-81	Mouse monoclonal	Millipore (MAB4381)	1:50	ICC, IHC, FC
Secondary antibodies				
Conjugate				
Goat anti-rabbit IgG	Alexa Fluor 555	Invitrogen (A31630)	1:500	ICC
Goat anti-rabbit IgG	Alexa Fluor 647	Invitrogen (A21245)	1:500	FC
Sheep anti-mouse IgG	FITC	Sigma (F2266)	1:128; 1:256*	ICC, FC*
Goat anti-mouse IgM	CY3	Millipore (AP128C)	1:200	ICC
Goat anti-mouse IgM	Alexa Fluor 488	Invitrogen (A21042)	1:100	FC

ICC, immunocytochemistry; IHC, immunohistochemistry; FC, flow cytometry.

*Indicates a different dilution used for FC.

Testis fixation and immunohistochemistry (IHC). Testes from two prepubertal and two sexually mature males were fixed, embedded in paraffin, and serial sections of 5-7 μm were produced for IHC. Briefly, testes were incubated overnight at rt in 5 mL modified Davidson's fixative (mDf; Electron Microscopy Sciences, Hatfield, PA, USA) per testis, bisected transversely, and incubated in fresh mDf for 24h. Then, tissue was submersed in 5 mL of 50% ethanol at rt for 1.5 h and stored in 5 mL of 70% ethanol for processing, embedding, and sectioning.

For IHC, adult and prepubertal testis sections were stained for KIT, GFRA1, GPR125, SSEA-1, SSEA-4, TRA-1-60, and TRA-1-81 (Table 1.1). After standard de-paraffinization and rehydration of tissue sections, a heat-induced epitope retrieval step using sodium citrate buffer (10 mM Sodium Citrate, 0.05% Tween20, pH 6.0-6.2) was performed in a pressure cooker for 3 min after rocking was initiated. For antigen detection, the EXPOSE Rabbit specific HRP/DAB detection IHC kit (Abcam #80437, Eugene, OR, USA) was used according to the manufacturer's protocol with minor modifications. Tissue sections were blocked with a protein solution provided in the kit for 10 min, washed, and incubated with primary antibodies at rt for 1.5 h. Tissue sections were then washed once with buffer solution and incubated 10 min with a rabbit anti-mouse secondary antibody, followed by 15 min with a Horseradish Peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody. Enzymatic development was completed with DAB (3, 3'-diaminobenzidine) chromogenic substrate solution until brown staining became visible, with incubation times ranging from 20 sec to 5 min. Stained sections were counterstained with hematoxylin for 30 sec, dehydrated, and a coverslip was applied with Permount (Thermo Fisher Scientific, Waltham, MA, USA). For nonspecific controls, the primary antibody was replaced with goat IgG serum diluted with the protein block. Staining was visualized with an Olympus BX60 microscope, and images captured with an Olympus DP72 digital camera.

Molecular characterization by qRT-PCR. The expression of cat pluripotent transcription factors *NANOG*, *POU5F1*, *SOX2*, the proto-oncogene *MYC*, and the internal standard *18SrRNA* genes were detected by qRT-PCR as previously described [55, 83] in sorted single-stained cells positive for SSEA-1 or SSEA-4 and subpopulations of cells double-stained for SSEA-4 and GFRA1 (SSEA-4+ only, GFRA1+ only, and dual positive cells). To obtain standards for analysis, five-fold

serial dilutions of feline mixed germ cell genomic DNA (10 ng/μL) in nuclease free water with 10 ng/mL tRNA were used to produce standard curves. Total mRNA was isolated from sorted cells using the RNeasy mini kit (Qiagen, Valencia, CA, USA) and reverse transcribed into cDNA using the QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA, USA) according to the manufacturer's directions. Primer sequences used for amplification of the target genes, cycling conditions, and Ct cutoff values for *MYC* (HQ846918), *NANOG* (EU366913), *POU5F1* (EU366914), and *SOX2* (HQ385804) were used as previously reported [55]. The *18SrRNA* human mRNA sequence (NM-X03205) was used as housekeeping gene [83]. Primers were tested for efficiency and amplification specificity by standard curve analysis and sequencing of PCR products. RT-PCR reactions were achieved by the addition of 1.75 μL of cDNA to 40 μL mastermix containing 30 μL 2X iQ SybrGreen Supermix (BioRad, Hercules, CA, USA) and 300 nM each of forward and reverse primer, with nuclease free water. Reactions were performed in duplicates of 25 μL each, with negative RT controls (reverse transcriptase omitted) to verify absence of genomic contamination, and a non-template control for each sample and gene, respectively. The cycling conditions were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 sec, 54.2 °C for 45 sec, and a final step of 72 °C for 5 min using a BioRad iQ5 multicolor real-time PCR system. Melt curves were carried out after the run to confirm single product amplification; all primers showed single product amplification.

qRT-PCR analysis. Technical replicates with standard deviation (sd) > 1.0 or with an average C_T above the cutoff value were omitted from analysis. The delta-delta C_T method was used for real time PCR data analysis. The C_T mean and sd were calculated for biological replicates, and data was normalized for different amounts of input cDNA using ΔC_T [C_T for the gene of interest (goi) minus C_T for the *18SrRNA* housekeeping gene (hk)] and the sd of the ΔC_T [$sd = (sd_{goi}^2 + sd_{hk}^2)^{1/2}$]. Next, $\Delta\Delta C_T$ was calculated by subtracting the ΔC_T of each sample from the ΔC_T of a reference cDNA sample (cat blastocysts). The sd of the $\Delta\Delta C_T$ is the same as the sd for the ΔC_T and is incorporated into final calculations to determine the n-fold increase or decrease in expression level of each gene in sorted SSCs, or $2^{-(\Delta\Delta C_T)}$, by substituting $\Delta\Delta C_T$ with $\Delta\Delta C_T + sd$ and $\Delta\Delta C_T - sd$.

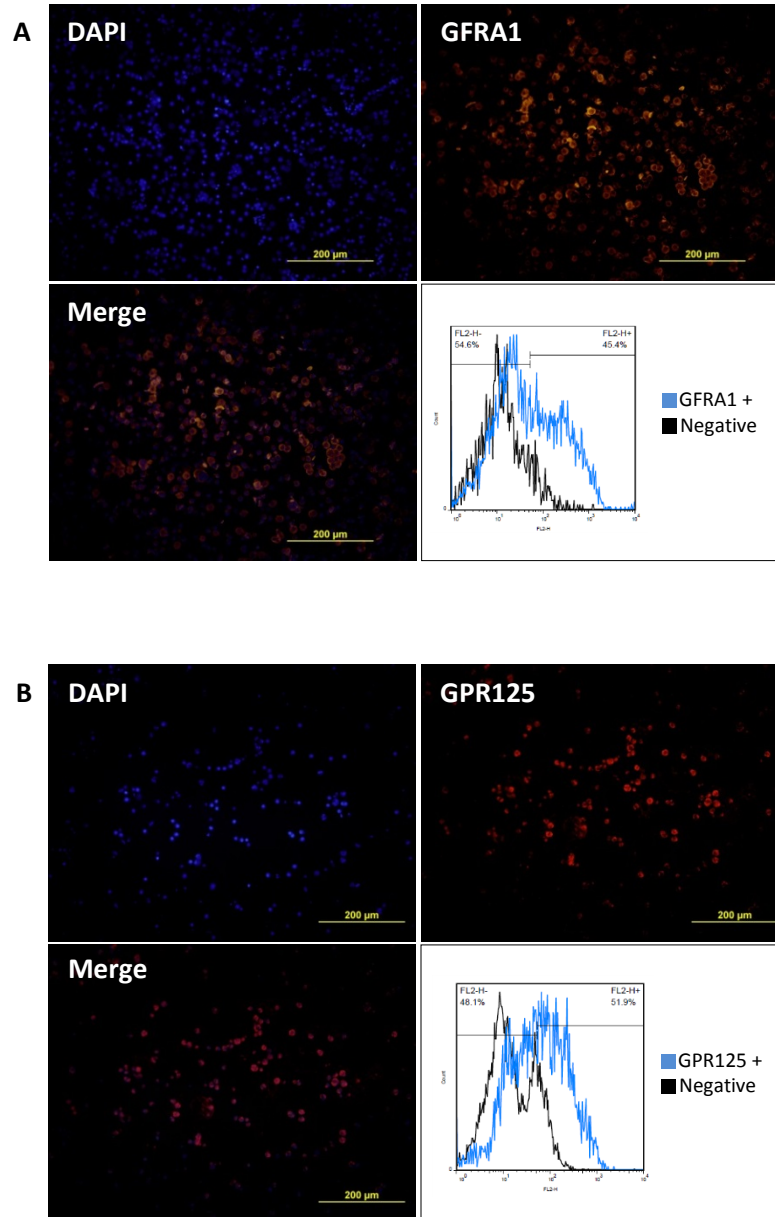
Results

Isolation of SSCs. To determine whether centrifugation of a single cell suspension of testicular cells in a density gradient can purify spermatogonial cells from other cell types, we processed adult and prepubertal testis and centrifuged digested single cells from seminiferous tubules over a 5-layer density gradient (35.0%, 30.0%, 27.5%, 25.0% and 20.0%) of Percoll. The mean number of cells/testis collected from adult cats ($n=21$) was $9.4 \times 10^6 \pm 7.1$, with $96.3\% \pm 6.0$ live cells, which was significantly higher than that of prepubertal cats ($n=6$; $3.9 \times 10^5 \pm 2.6$ total cells with $96.2\% \pm 2.6$ live cells). Centrifugation of single cells over the 5-density gradients successfully purified populations of mixed germ cells from cell debris, red blood cells, and sperm. Mixed germ cells were found in the bands formed at the 30.0%, 27.5%, and 25.0% layers, while cell debris was found mostly at and above the 20.0% band, and red blood cells and sperm were pelleted under the 35.0% layer. Spermatogonial cells, found within the mixed germ cell populations, were morphologically identified by their characteristic large nucleus with a homogenous appearance in the bands of mixed testicular cells (Fig 1.2B).

Phenotypic characterization of cat SSCs. To explore phenotypic characteristics of cat SSCs, we assessed the fluorescence expression of SSC markers, commonly used in other mammalian species, ITGA6, CD9, GFRA1, GPR125, and THY1, differentiation marker KIT, and pluripotent markers SSEA-1, SSEA-4, TRA-1-60, and TRA-1-81, on a population of cat mixed germ cells purified by gradient densities of Percoll.

Analysis by ICC and FC revealed that a large portion of cells were positive for the SSC markers GFRA1 ($44.7 \pm 2.5\%$) and GPR125 ($50.0 \pm 3.4\%$) and the differentiation marker KIT ($59.2 \pm 5.3\%$; Fig 1.3), but fluorescence was not detected for ITGA6, CD9, and THY1. Moreover, analysis by FC detected a low number of cells positive for the pluripotent markers TRA-1-60 ($18 \pm 4.8\%$), TRA-1-81 ($16.3 \pm 4.5\%$), and SSEA-1 ($15.4 \pm 7.1\%$), and fewer cells were positive for SSEA-4 ($5.9 \pm 2.9\%$; Fig 1. 4).

Figure 1.3. Mixed germ cells were stained with primary antibodies specific for common SSC-surface markers GFRA1 (A) and GPR125 (B) and differentiation marker KIT (C). Nuclei were stained with DAPI. Histograms show the shift in fluorescence intensities from control samples (black) to stained samples (blue), and percentages indicate the % positive and % negative cells within the stained sample.



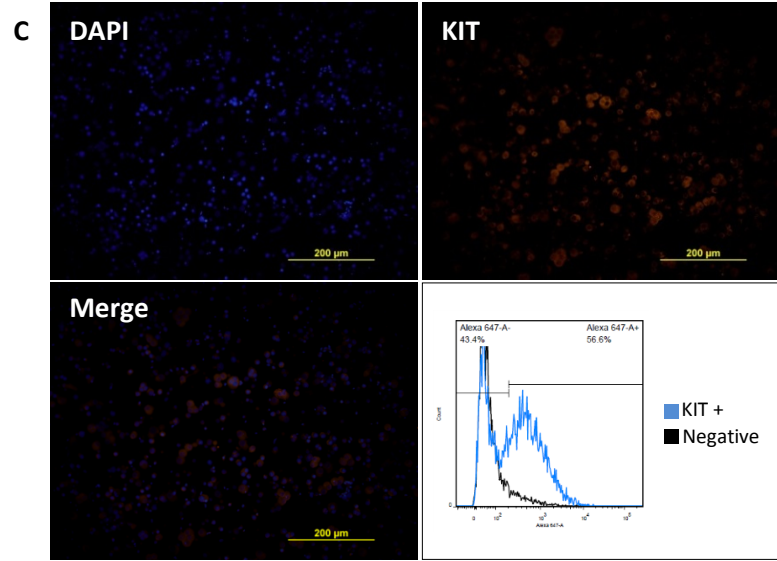
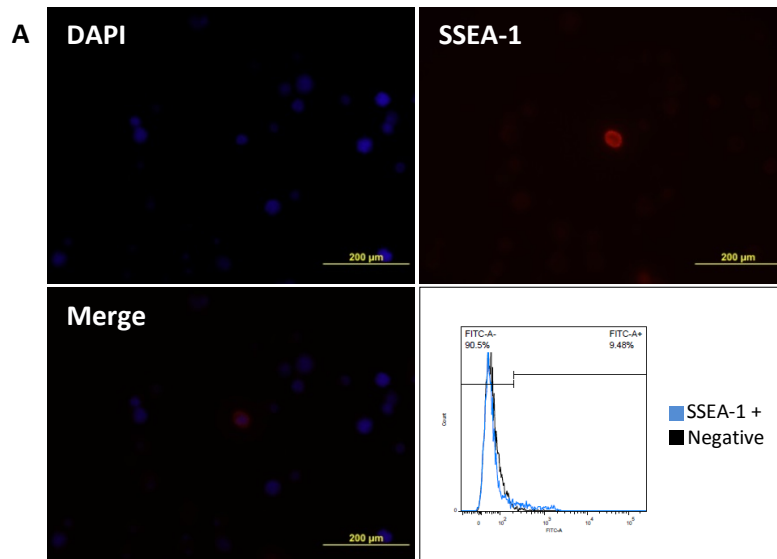
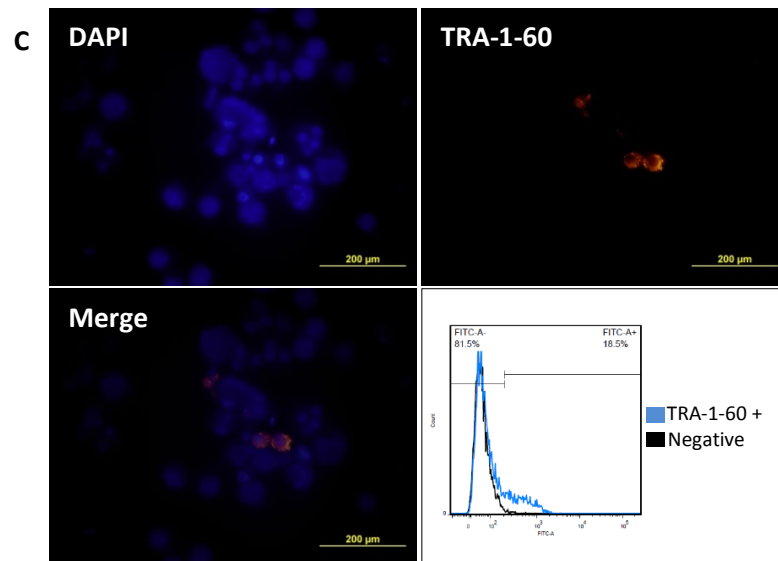
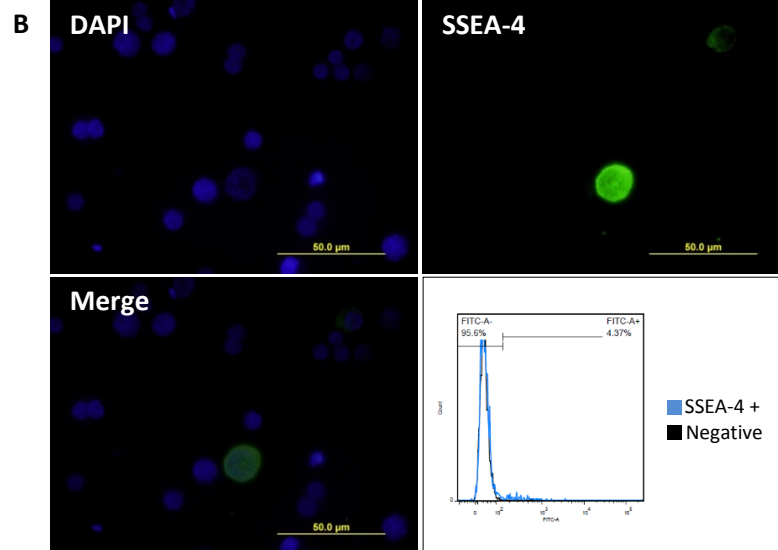
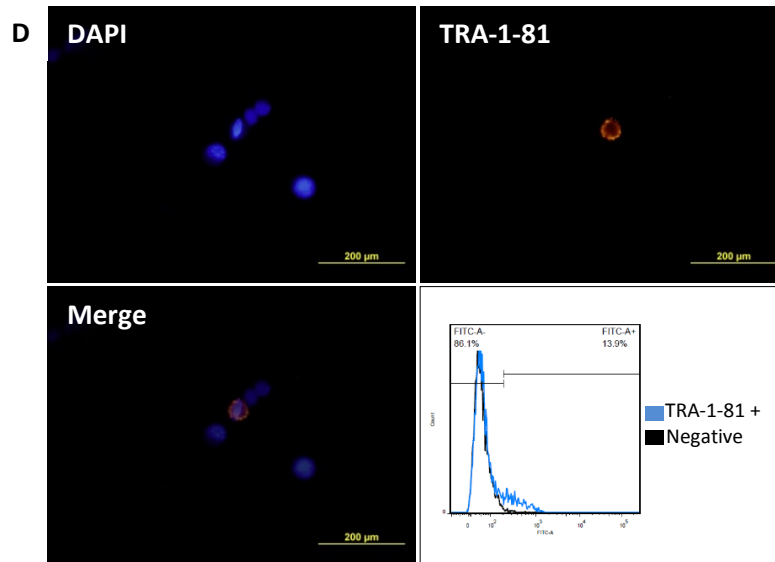


Figure 1.4. Mixed germ cells were stained with primary antibodies specific for pluripotent markers SSEA-1 (A), SSEA-4 (B), TRA-1-60 (C), and TRA-1-81(D). Nuclei were stained with DAPI. Histograms show the shift in fluorescence intensities from control samples (black) to stained samples (blue), and percentages indicate the % positive and % negative cells within the stained sample.







Then, we examined the distribution of cells positive for these markers within adult and prepubertal seminiferous tubules. Analysis by IHC showed that cells from adult testis expressing GFRA1 and GPR125 were at various stages of spermatogenesis across the seminiferous tubules (Fig 1.5), while pluripotent markers, SSEA-1, SSEA-4, TRA-1-60, and TRA-1-81, in prepubertal and adult tissue sections, were localized at the basement membrane of the seminiferous tubules (Fig 1.6). Interestingly, SSEA-4 was expressed only in single cells, whereas SSEA-1, TRA-1-60, and TRA-1-81 were found in pairs and chains of spermatogonia. In addition, KIT was not detected in tissue sections by IHC, regardless of previous detection by ICC and FC (Fig 1.5).

Figure 1.5. Adult cat testis tissue stained for common SSC-surface markers GFRA1, GPR125, and KIT. Brown staining with DAB (3, 3'-diaminobenzidine) chromogenic substrate solution indicates positive cells. Tissue was counterstained with hematoxylin to reveal nuclei.

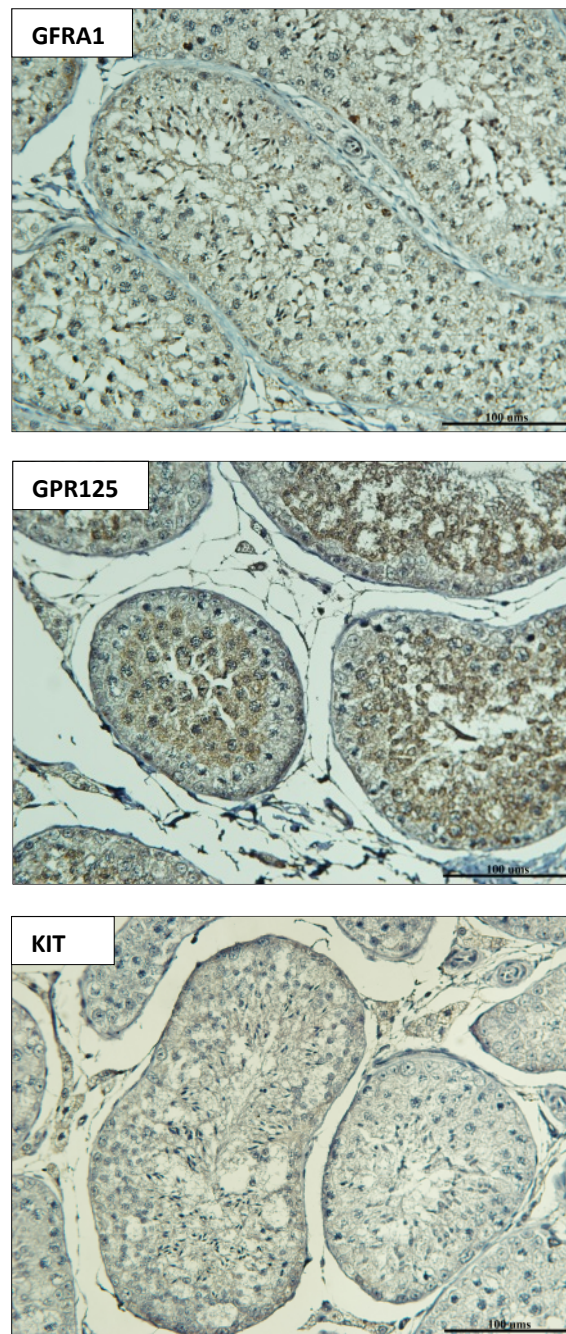
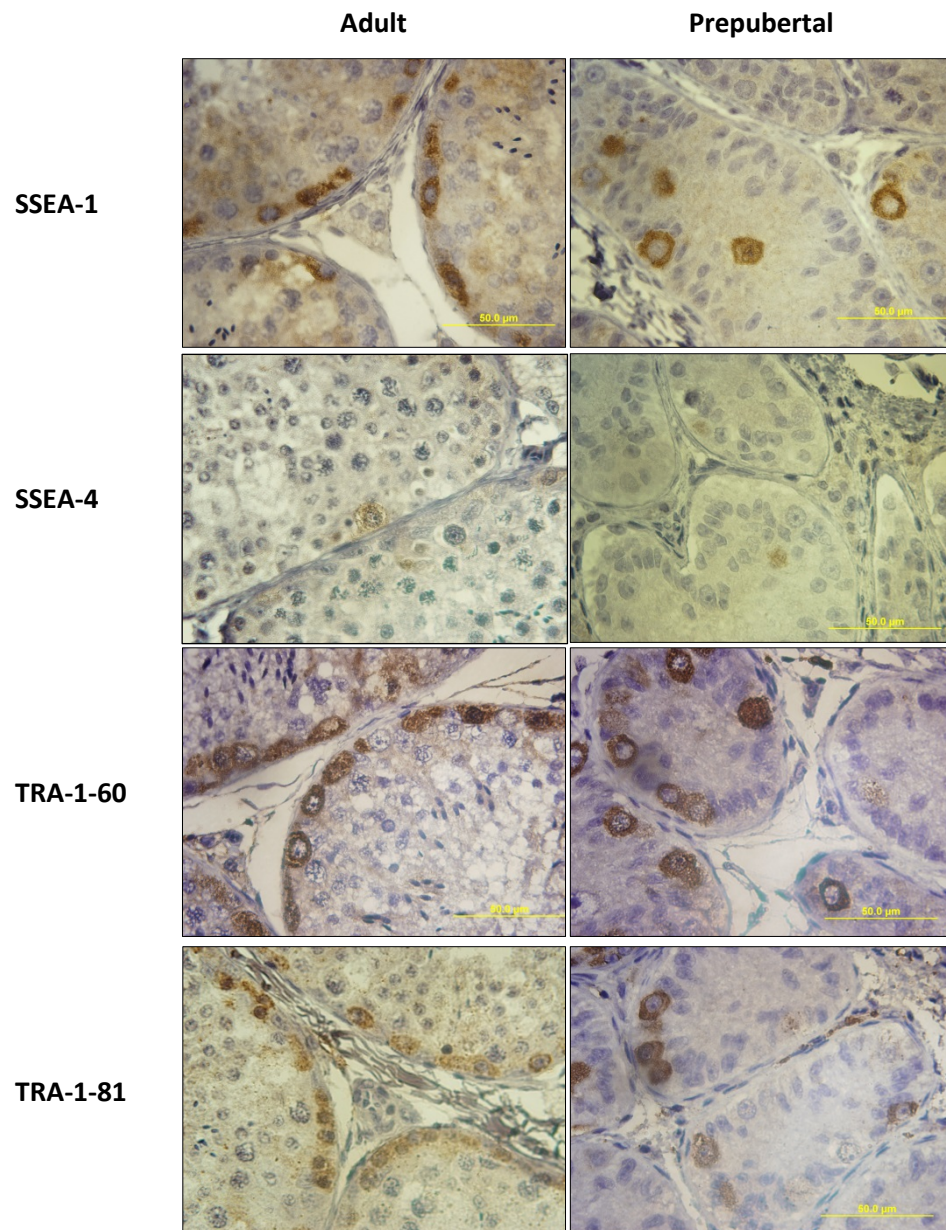
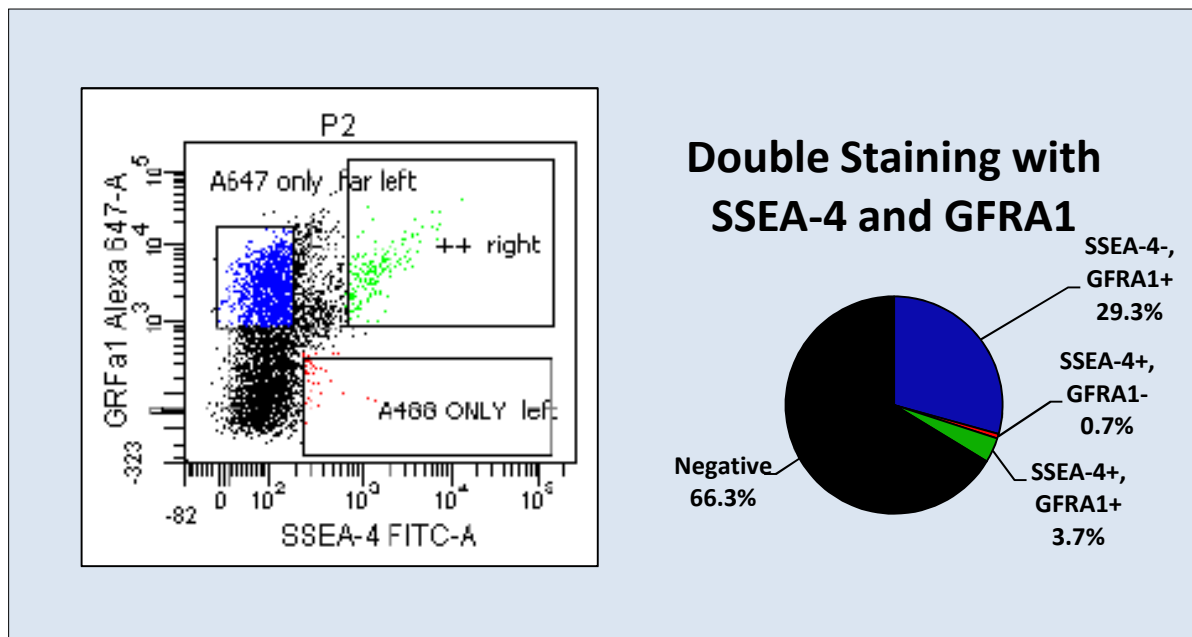


Figure 1.6. Adult and prepubertal testis tissue stained for pluripotent markers SSEA-1, SSEA-4, TRA-1-60, and TRA-1-81. Brown staining with DAB (3, 3'-diaminobenzidine) chromogenic substrate solution indicates positive cells. Tissue was counterstained with hematoxylin to reveal nuclei.



To further characterize SSEA-4+ cells and investigate the use of SSEA-4 as a surface marker for isolation of cat SSCs, pools of mixed germ cells were double stained and quantified using the SSEA-4 and GFRA1 antibodies. Analysis by FC identified three distinct cell subpopulations that were positively stained for: GFRA1+ only ($29.3 \pm 17\%$), SSEA-4+ only ($0.7 \pm 0.5\%$), and a subpopulation co-localizing GFRA1 and SSEA-4 ($3.7 \pm 2.8\%$; Fig 1.7).

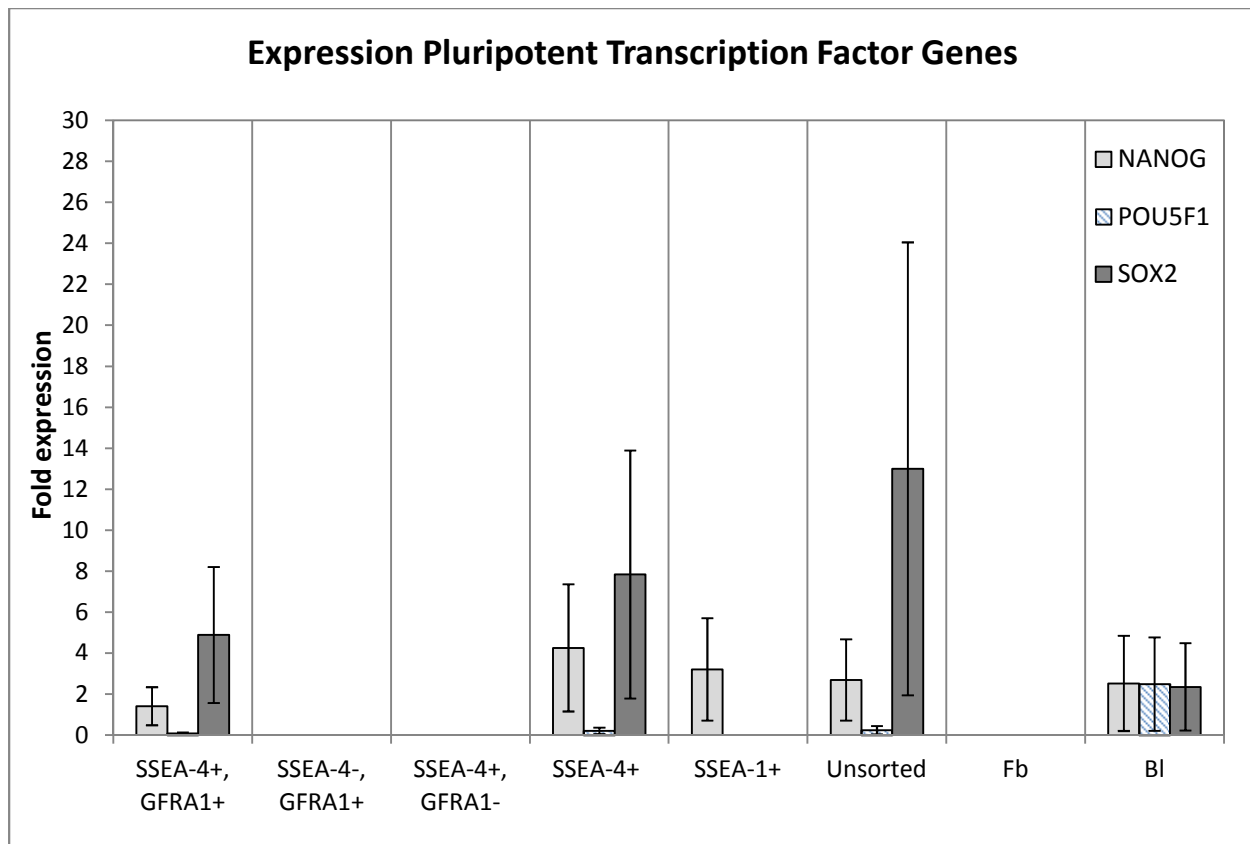
Figure 1.7. Flow cytometry analysis of mixed germ cells double stained for the pluripotent marker SSEA-4 and the common SSC marker GFRA1. Distinct populations of SSEA-4+, GFRA1- ($0.7 \pm 0.5\%$) and SSEA-4-, GFRA1+ ($29.3 \pm 17\%$) positive cells were revealed in addition to a population of cells that expressed both SSEA-4 and GFRA1 ($3.7 \pm 2.8\%$).



Molecular characterization of cat SSCs. To explore the undifferentiated stage of cat SSCs, we evaluated the expression of pluripotent transcription factors *NANOG*, *POU5F1* and *SOX2* and the proto-oncogene *MYC* by qRT-PCR in unsorted and sorted cells single-stained for SSEA-1+ or SSEA-4+, and subpopulations of sorted cells double-stained for SSEA-4 and GFRA1 (SSEA-4+, GFRA1-; SSEA-4-, GFRA1+; and SSEA-4+, GFRA1+). In addition to analyzing RNA from cat spermatogonial subpopulations, we included RNA from blastocysts – derived *in vitro* – at day 8 after IVF (pool of 3-5 embryos) and cat embryonic fibroblasts ($\sim 2 \times 10^6$ cells). Relative transcript abundance for each of the genes analyzed is shown in Fig 1.8. qRT-PCR analysis revealed expression of transcription factors *NANOG*, *POU5F1*, and *SOX2* in sorted single-stained

SSEA-4+ cells, double-stained cells positive for both SSEA-4+ and GFRA1+ and unsorted cells. Single-stained SSEA-1+ cells expressed only *NANOG*, and double-stained cells positive only for SSEA-4+ or GFRA1+ did not express any of the transcription factors. Expression levels of *NANOG* in cat SSCs are similar to that in blastocysts, while *POU5F1* transcript levels were lower. In contrast, *SOX2* is upregulated (13 fold for unsorted cells, 7.8 fold for sorted single-stained SSEA-4+ cells, and 4.9 fold for double-stained sorted cells positive for both SSEA-4+ and GFRA1+) to that of blastocysts (2.3 fold). Expression of *MYC* was detected in all cell types, with all populations except for SSEA-1+ cells showing higher levels (4.45 fold to 36.36 fold) of *MYC* than the levels in blastocysts (2.23 fold; data not shown).

Figure 1.8. Fold expression of *NANOG*, *POU5F1*, and *SOX2* genes in cat SSCs: 1) for dual-stained sorted cells positive for both SSEA-4+ and GFRA1+ , GFRA1+ only and SSEA-4+ only, and for 2) single-stained sorted cells for SSEA-4+ and SSEA-1+, 3) unsorted cells, 4) fibroblasts (Fb) and 5) in vitro-derived blastocysts (Bl). Bars indicate range of expression determined using the standard deviation of $\Delta\Delta Ct$.



Discussion

The ability to enrich a cell population of SSCs from mixed germ cells offers the possibility of enhancing colonization and spermatogenesis after transplantation, as well as a better understanding of the mechanisms of spermatogenesis during *in vitro* culture. Toward these goals, we identified surface markers SSEA-1, SSEA-4, TRA-1-60 and TRA-1-81, which are expressed in pluripotent embryonic stem cells, as positive markers for isolating spermatogonial subpopulations from other testicular cells in the domestic cat. In contrast to other SSC surface markers, GFRA1 and GPR125 also were expressed in differentiated cat spermatogonial cells. Moreover, the lower number of cells positive for the pluripotent markers and their restriction to cells located near the basement membrane of the seminiferous tubule indicates that pluripotent surface markers are specific to undifferentiated spermatogonia in the cat.

For enriching SSC suspensions, the differentiation surface marker KIT has been a valuable marker for removing differentiated germ cells. However, other surface markers have been also used to isolate SSCs in several species, including mice and humans. In domestic cats, undifferentiated type A spermatogonia express the GFRA1 marker [13], and some cells expressing GFRA1 are localized at the basement membrane of the seminiferous tubules and express genes specific for SSC, *GFRA1* and *ZBTB1* [73]. These studies suggest that GFRA1 marker may be a useful tool for purifying SSCs. It is known that SSC markers are primarily expressed in spermatogonial cells; however, in some mammalian species, they can also be expressed in somatic or differentiating germ cells in the testis. For example, in rodents, CD9 is specifically expressed in pairs or chains of undifferentiated spermatogonia [46], but also co-expressed with KIT in differentiating spermatogonia [45]. In humans, GFRA1 is detected not only in spermatogonia cells, but also in Sertoli and Leydig cells [30, 49]. Similarly, in the present study, we observed that GFRA1 and GPR125 were non-specific to cat SSCs as indicated by the high number of positive cells detected by FC and localization in the testis. Immunolocalization confirmed that GFRA1 and GPR125 were also expressed in differentiated germ cells distributed throughout the seminiferous tubules. Our results clearly indicated the heterogeneity among the pool of single spermatogonial cells and how the use of GFRA1 and GPR125 markers may lead to erroneous identification of cat SSCs [77].

It is not clear why cat mixed germ cells were negative to surface antigen markers CD9, ITGA6, and THY1, but it is possible that differences among species are influencing the results. In fact, variable expression of surface markers between rodent and human SSCs has been reported. However, the negative reactivity of mixed germ cells to CD9, ITGA6, and THY1 may be due partially to the type of antigen epitope recognized by the antibody, which may be different between the human and mouse and the cat.

Variable expression detected by antibodies can be observed with different staining techniques, in particular with IHC. In a previous study in which the pattern of KIT expression on cat tissues by IHC was evaluated, cat testis tissue did not react to KIT, regardless of different intensity reaction in other cell types and neoplastic tissues [69]. Likewise, in the present study, we did not detect positive KIT cells in cat testis tissue staining by IHC, even though flow cytometry, using the same antibody, revealed that at least 50% of mixed germ cells were positive for KIT. The reason(s) for the discrepancies between IHC and FC findings are not clear; however, the lack of KIT staining in cat testis may be due partially to the reagents used in IHC that may have interfered with antigen detection.

During the last few years, characterization of SSCs with pluripotent surface markers in different mammalian species has progressed. Rodent SSCs express SSEA-1 [81], while SSCs from human and non-human primates, including Rhesus macaque (*Macaca mulatta*) and the common marmoset (*Callithrix jacchus*), expressed SSEA-4 [20, 32, 74, 80, 82] but not SSEA-1 [84]. In addition to SSEA-4, TRA-1-60 and TRA-1-81 were detected in spermatogonia of non-human primates [80]. In contrast, in the present study, we observed that cat spermatogonial cells expressed all four antigens (SSEA-4, SSEA-1, TRA-1-60 and TRA-1-81). Although these antigens were present in both prepubertal and adult cat testis tissue, indicating a likely co-expression in the earlier type A spermatogonia which are the only germ cells present in the prepubertal testis, the expression patterns in adult tissue revealed a change in phenotype with differentiation. In fact, in adult cat testis, SSEA-4 was detected only in a low number ($5.9 \pm 2.9\%$) of single cells, whereas SSEA-1 ($15.4 \pm 7.1\%$), TRA-1-60 ($18 \pm 4.8\%$), and TRA-1-81 ($16.3 \pm 4.5\%$) were further expressed in pairs and chains of spermatogonia, about half of which will differentiate into sperm [18-20]. Therefore, based on these results, we suggest that expression

of SSEA-4 is likely restricted to SSCs and single type A spermatogonia, and expression is lost as cells differentiate.

Although the biological significance of SSCs positive for SSEA-4 is not clear, in humans, SSEA-4 is an optimal marker for isolating active SSC subpopulations [20, 32]. In fact, cell populations enriched for SSEA-4 have higher proliferation activity, colonization ability, and expression levels of SSC-specific genes, including pluripotent stem cell marker *POU5F1*, as compared to populations that are sorted with other surface markers [20, 32]. In addition, SSEA-4+ cells not only maintained their undifferentiated state and gene expression pattern during long-term culture, but were able to be maintained in culture for more passages than the populations obtained with other SSC markers [32]. Interestingly, a portion of SSEA-4+ cells co-expressed with cells positive for ITGA6, GPR125, or GFRA1 [20, 32]. Similarly, in the Rhesus monkey, a subpopulation of ITGA6+, THY1+, and KIT- (triple stained) cells were also SSEA-4+ (24%) [74]. These findings indicate that various subpopulations of SSCs may exist, each expressing different combinations of SSC and pluripotent markers, and some may be functional, actively dividing subpopulations, while others may be quiescent [74].

Likewise, we observed that the percentage of SSEA-4+ cells (~6%) fell within the same range of SSEA-4+ cells reported in the monkey (~2%) [74] and the human (~13%) [20]. After sorting cells by FACS with dual-staining for SSEA-4 and GFRA1, a subpopulation of cat SSEA-4+ cells co-expressed with GFRA1+ ($3.7 \pm 2.8\%$) was identified, and this specific subpopulation of sorted cells expressed the pluripotent transcription factors *NANOG*, *POU5F1*, and *SOX2*. Analysis of SSEA-4+ cell population sorted only for SSEA-4 (without isolation of GFRA1+), showed that all three pluripotent transcription factors were also present and in higher levels than the subpopulation of cells positive for both SSEA-4 and GFRA1. The biological significance of the co-expression of SSEA-4 and GFRA1 is not clear. However, although our results showed that the GFRA1 marker was not specific to spermatogonia in the cat, GFRA1 has an important role in regulating the proliferation and self-renewal of SSCs [18, 30-32, 47, 48, 85]. Moreover, expression of pluripotent transcription factors was not found in subpopulations of SSEA-4-, GFRA1+ cells or SSEA-4+, GFRA1- cells sorted by dual-staining, indicating that loss of the dual positive phenotype may lead to loss of their self-renewal ability, and they go into cell

differentiation. Therefore, we interpret our results to indicate that subpopulations of cat SSEA-4+ cells localized at the basement membrane of the seminiferous tubules and expressing pluripotent transcription markers are likely SSCs and that SSEA-4 is an ideal surface marker for selecting cat SSCs.

In summary, our results show that markers commonly used for SSC identification in other species may be less reliable for isolating cat SSCs, while pluripotent markers, particularly SSEA-4, may provide more enriched SSC populations. SSCs are low in number in the testis, and the smallest subpopulation of spermatogonial cells that we identified was SSEA-4+ and also found to express *NANOG*, *POU5F1*, and *SOX2*. Although the other pluripotent surface markers investigated here may be expressed by SSCs, the large percentage of positive cells and formation of chains indicates they are likely not SSC-specific, but may be characteristic of other stages of spermatogonia. Double staining with SSEA-4 and GFRA1 and RT-PCR analysis confirmed that, as with Rhesus monkey and human, SSCs in the cat may be only a subpopulation of the SSEA-4+ cells. Future studies to examine and compare the colonization potential of these subpopulations to determine their biological roles are warranted.

CHAPTER 2

SUCCESSFUL COLONIZATION OF SSEA-1+ AND SSEA-4+ DOMESTIC CAT SPERMATOGENIAL SUBPOPULATIONS AFTER TRANSPLANTATION INTO PREPUBERTAL MALES WITHOUT DEPLETION OF ENDOGENOUS GERM CELLS

Abstract

Spermatogonial stem cell transplantation (SSCT) in the domestic cat (*Felis catus*) may be a promising approach for preservation of endangered felids, as demonstrated by the successful transplantation of cat and ocelot (*Leopardus pardalis*) SSCs into the testes of cats which resulted in donor sperm production [13]. Studies in other mammalian species indicated that transplantation of purified SSC populations and elimination of recipient endogenous germ cells prior to SSCT may improve donor cell colonization efficiency [20, 30-32]. We previously identified the pluripotent markers stage specific embryonic antigen-1 and -4 (SSEA-1 and SSEA-4) as plausible surface markers for cat SSCs [78, 79]. Therefore, donor cell suspensions of unsorted mixed germ cells and SSEA-1+ and SSEA-4+ spermatogonial subpopulations, isolated by fluorescence activated cell sorting, were obtained for comparing colonization ability and labeled with the red fluorescent membrane dye PKH26 for detection within recipient testes. To circumvent the use of chemical or radiation treatment to eliminate endogenous germ cells, we evaluated the suitability of prepubertal male cats that were five and six months old. Recipients were castrated 10-12 weeks post-SSCT. Half of each testis was enzymatically digested to identify donor cell colonies within the seminiferous tubules, and half was snap-frozen to evaluate cross-sections for spermatogenic progression of donor cells. Colonies were detected in one five month-old recipient of SSEA-1+ cells, one five month-old and one six month-old recipient of SSEA-4+ cells, and both six month-old males injected with mixed donor cells. Although sperm was present in the epididymides, there was no evidence of donor sperm. Our results demonstrated that purification of SSCs with surface markers specific for SSCs is not necessary for successful colonization; however, SSEA-4 and SSEA-1 are ideal surface markers for

purifying subpopulations of SSCs from mixed germ cell populations in the cat. Although SSCs were able to colonize the seminiferous tubules of prepubertal males without depletion of endogenous germ cells, it is possible that the low number of endogenous germ cells may decrease colonization efficiency.

Introduction

Most species of felids are listed as threatened or endangered. Even though captive breeding and assisted reproductive technologies (ARTs) have had varied success depending on species, conservation efforts are further hindered by the deaths of genetically important animals. A limited amount of sperm may be acquired post-mortem but only from fertile, sexually mature males. New alternative ARTs are requisite to circumvent the limitations inherent in epididymal sperm collection and storage.

Spermatogonial stem cells (SSCs) are progenitor cells capable of both self-renewal and differentiation into sperm. Spermatogonial stem cell transplantation (SSCT) was first introduced in 1994 by Brinster and Zimmerman [21]. In most early studies, mice were used as recipients of donor SSCs, regardless of donor species, to confirm that isolated cells contained SSCs capable of colonization. Syngenic transplantation (within the same-species) of mouse [21, 27, 42], canine [29], porcine [37, 38] and bovine [23] SSCs restored spermatogenesis in host recipients. However, xenogenic transplantation of cells from non-murine species, with the exception of some other rodents, did not result in establishing spermatogenesis despite colonization of the murine seminiferous tubules [9, 28, 33]. The lack of spermatogenesis in xenogenic transplantation may be attributed to phylogenetic distance between donor and recipient species. On the other hand, in a recent study, xenogenic transplantation of ocelot mixed germ cells into domestic cat testes produced colonization and differentiation through spermatogenesis into spermatozoa [13]. These results showed the potential use of the domestic cat for xenogenic transplantation of other felid SSCs.

Isolation and enrichment of SSCs play an important role in the successful colonization and spermatogenesis after transplantation. In the first report on transplantation of mixed germ cells from a wild felid into domestic cat testis, germ cells were isolated from the donor testis by enzymatic digestion and enriched by differential plating and density gradient separation [13].

Although successful colonization and spermatogenesis were observed, low numbers of ocelot spermatozoa were found in the duct of the domestic cat epididymis. Relatively few SSCs are present in the testis [27, 28], and, possibly, increasing the numbers of SSCs by separating them from somatic cells and other stages of germ cells may allow for increased colonization and spermatogenesis efficiency [27]. Methods used to purify SSCs include immunostaining with surface markers expressed only by SSCs and isolated by fluorescence activated cell sorting (FACS) [20, 77] or magnetic activated cell sorting (MACS) [20, 30-32]. The surface marker glial cell line-derived neurotrophic factor receptor alpha 1 (GFRA1) has been detected in cat undifferentiated type A spermatogonia [13, 73]. Although the SSC markers are expressed primarily in spermatogonial cells, in some species they can be expressed in somatic or differentiating germ cells in the testis. Recently, we demonstrated that markers commonly used for SSC identification in other mammalian species may be less reliable for isolating cat SSCs, while pluripotent markers, particularly stage-specific embryonic antigen-4 (SSEA-4), may provide enriched SSCs populations [78, 79].

Conventionally, male recipients of SSCs are treated before transplantation to deplete endogenous germ cells, which consequently will reduce competition with transplanted SSCs, and provide an open niche for donor cells to colonize and prevent contamination of potential donor sperm by recipient sperm. Injection of busulfan, a cytotoxin commonly used to destroy cancer cells [17, 21, 33, 34], is the most common method of depleting endogenous germ cells in small animals, while focal irradiation of the testes is used frequently in large mammals [9, 13, 23, 29]. In adult domestic cats, both methods, treatment with busulfan and focal radiation, have been used to deplete endogenous germ cells [9, 13]. Even though both methods significantly reduced the percentage of endogenous germ cells, busulfan is toxic and endogenous spermatogenesis was restored after thirteen weeks post-radiation [13]. Few alternatives have been investigated, including the employment of sexually immature males with fewer endogenous cells present [27, 38, 39, 41].

In the present study, we determined whether transplantation of a purified subpopulation of domestic cat spermatogonial cells positive for the pluripotent markers SSEA-1 or SSEA-4 enhanced colonization and spermatogenesis after SSCT as compared to transplanting

a mixed germ cell population. We also evaluated the use of sexually immature male cats as an alternative to subjecting the recipients to toxic drugs or radiation.

Materials and Methods

Subjects. Domestic cats used as SSC recipients were group housed in environmentally controlled rooms at the Audubon Center for Research of Endangered Species (ACRES). Fresh food was provided daily and water always available. All animal procedures were approved by the Institution Animal Care and Use Committee of ACRES as required by the Health Research Extension Act of 1985 (Public Law 99-1580).

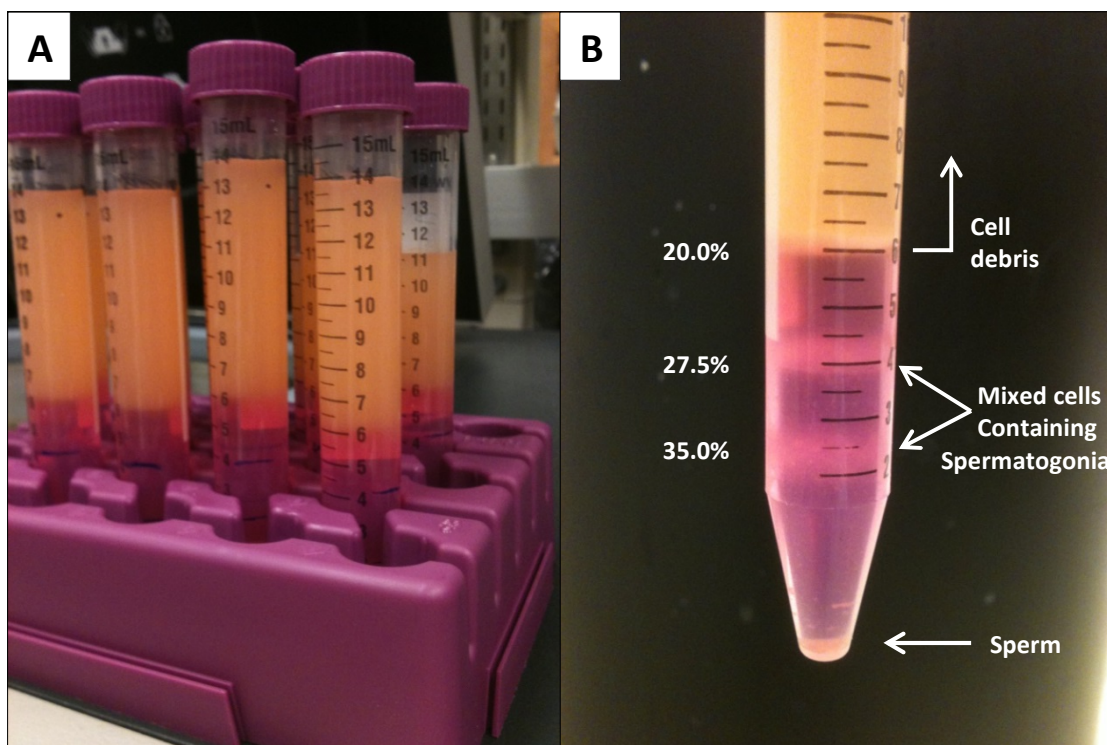
Chemicals. All chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

Tissue procurement and handling. Testes from sexually mature domestic cats were obtained after routine castration from ACRES (n = 2) and local veterinary clinics (n = 92). Tissue was transported at ambient temperature in HEPES buffered saline solution (Ultrasaline A Solution, Bio-Whittaker, Walkersville, MD, USA) and either processed immediately or stored up to two days at 4°C. Testes were rinsed in Hanks' Balanced Salt Solution (HBSS), and the tunica vaginalis and epididymal tissue were carefully trimmed to minimize damage to the underlying tissue. Testes were then rinsed in fresh HBSS and weighed.

Mixed germ cell isolation. Seminiferous tubules were exposed by removing the tunica albuginea, and tubule tissue was incubated for 30-45 min in HBSS supplemented with 25 µg/mL amphotericin-B, 10 µg/mL streptomycin, and 50 µg/mL gentamicin. Mixed testicular cell suspensions were obtained using a two-step enzymatic digestion, as previously described [9], followed by dual filtration through 100 µm and 40 µm nylon mesh filters (BD Falcon, Tewksbury, MA, USA), and Percoll density gradient centrifugation. Briefly, Percoll was diluted to gradient concentrations of 35.0%, 27.5% and 20.0% with HBSS, and each gradient placed in a 15 mL tube, starting with the most concentrated gradient at the bottom of the tube (Fig 2.1). Then, filtered cell suspensions were slowly added to the top layer of the Percoll gradient, and centrifuged at 725 x g for 25 min. Cell suspensions containing spermatogonial cells, identified by their morphology, formed bands at the 27.5% and 35.0% Percoll layers. For differential cell adhesion culture, cells found in these two bands were pooled, rinsed three times with

Dulbecco's phosphate buffered saline (DPBS), and re-suspended in DMEM/F12 medium (Gibco, Frederick, MD, USA) supplemented with 15% Knockout Serum Replacement (KOSR; Invitrogen/Life Technologies, Carlsbad, CA, USA), 5% FBS (Hyclone/GE Healthcare Life Sciences, Piscataway, NJ, USA), 1 mM L-glutamine, 0.1 mM β -mercaptoethanol, 1.25% non-essential amino acids, 12.5 μ g/mL amphotericin-B, 10 μ g/mL streptomycin, and 50 μ g/mL gentamicin, and cultured overnight at 38°C in a humidified atmosphere of 5% CO₂ in air.

Figure 2.1. Density gradient separation of mixed germ cells. Mixed germ cell suspensions were layered over a Percoll density gradient of 20.0%, 27.5%, and 35.0% Percoll (A). Mixed cells containing spermatogonia were collected from the 27.5% and 35.0% bands (B).



Cell sorting by flow cytometry (FACS). Isolation of SSCs from mixed germ cells was performed by FACS after incubation with primary mouse anti-SSEA-1 (Millipore, Billerica, MA, USA) or mouse anti-SSEA-4 (Millipore, Billerica, MA, USA) antibodies. Briefly, after overnight cell culture for differential cell adhesion, cells suspended at, but not adhered to, the bottom of the tissue culture dish were collected, filtered through a 40 μ m nylon mesh filter, equally divided into two 50 mL conical tubes, and washed twice with DPBS by centrifugation at 220 x g

for 5 min. Cell pellets were re-suspended to a concentration of 10×10^7 cells and incubated for 45 min on ice with 5 mL of a blocking solution [1% bovine serum albumin (BSA) and 5% goat or 5% sheep serum] to inhibit unspecific antibody binding. Then, cells were incubated with 4 mL of primary antibody anti-SSEA-1 (1:50 dilution), or 2.5 mL of primary antibody anti-SSEA-4 (1:50 dilution) for 1 h on ice. After one wash with PBS + 1% BSA, cells were incubated for 45 min on ice to detect SSEA-1 with secondary goat anti-mouse IgM Alexa Fluor 488 conjugated (1:100 dilution; Invitrogen/Life Technologies, Carlsbad, CA, USA), and SSEA-4 with secondary sheep anti-mouse IgG conjugated with fluorescein isothiocyanate-FITC (1:256 dilution; Invitrogen/Life Technologies, Carlsbad, CA, USA). After incubation, cells were washed once with PBS + 1% BSA, and pellets were re-suspended in DMEM/F12 medium (to a concentration of 10×10^6 cells/mL) on ice until sorting. Controls for nonspecific auto-fluorescence were performed by replacing the primary antibody with goat or sheep IgG and cultured with secondary antibody. Unstained cells were also used as controls. Cells were sorted using two flow cytometers (FACSCalibur and FACSARIA, Becton Dickinson Biosciences, San Jose, CA, USA) with parameters set using the software FACSDiva (Becton Dickinson Biosciences, San Jose, CA, USA). Sorted cells, positive for each of the markers, were collected into 1 mL DMEM/F12 medium supplemented with 20% KOSR, 1 mM L-glutamine, 0.1 mM β -mercaptoethanol, 1.25% non-essential amino acids, 6 μ M/mL Y27632 ROCK-inhibitor (Stemgent, Cambridge, MA, USA), 12.5 μ g/mL amphotericin-B, 10 μ g/mL streptomycin, and 50 μ g/mL gentamicin and cultured at 38°C in a humidified atmosphere of 5% CO₂ in air for 24 h.

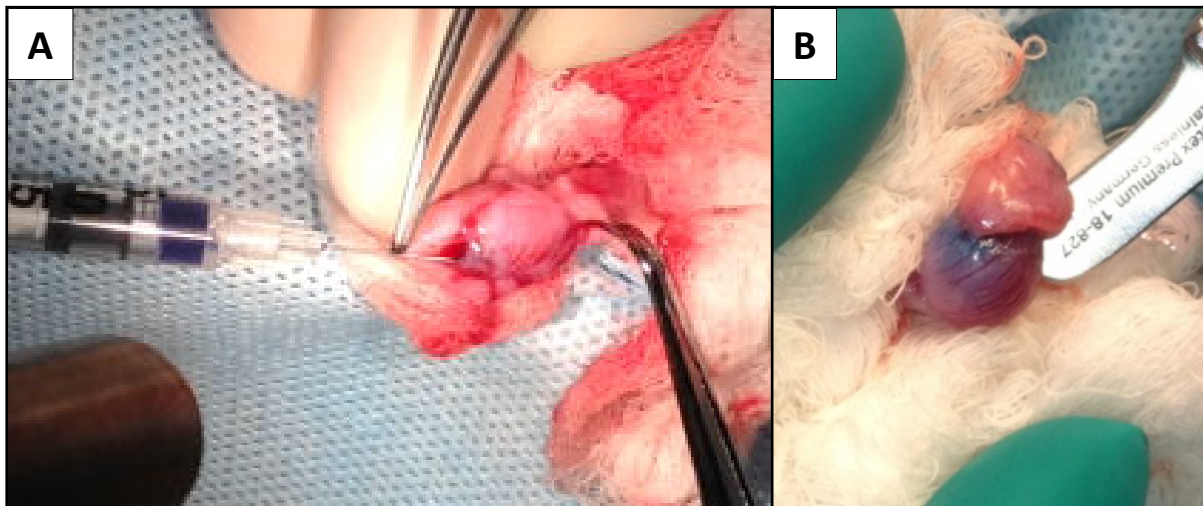
Cell membrane staining with PKH26 fluorescence linker. To identify transplanted donor cells in testis of recipient cats, cell membranes were labeled before transplantation with PKH26 Red Fluorescent Cell Linker Mini Kit, following manufacturer's guidelines with minor modifications. Briefly, sorted cells, ranging from 2×10^5 to 8×10^5 , were centrifuged in DPBS at 310 x g for 5 min, and supernatant was discarded. Resultant pellets were re-suspended in 250.5 μ L of Diluent C (125 μ L; PKH26 Fluorescent Cell Linker Kit) and a dye solution (125 μ L Diluent C + 0.5 μ L PKH26), mixed by gentle pipetting, and incubated for 5 min at room temperature. To bind the excess dye, an equal volume of DMEM/F12 medium was added to the stained cells, and the mixture was cultured for 1 min, washed and re-suspended in a final

volume of 100 to 200 μ l of DMEM/F12. For labeling the large number of mixed germ cells (20×10^6), reagent volumes were scaled upward, using 2.004 mL of Diluent C (1 mL) and a dye solution (1 mL Diluent C + 4 μ l PKH26) for staining. Stained cells were evaluated for PKH26 fluorescence using an Olympus IX71 microscope and images were taken with an Olympus DP71 digital camera.

Transplantation of mixed germ cells and SSCs into prepubertal male cat recipients.

Mixed germ cells (unsorted cells), and SSCs sorted by the expression of SSEA-1 and SSEA-4, were transplanted into the external rete testis of five-month-old ($n = 6$) and six-month-old ($n = 6$) prepubertal male recipients. For SSCT, each testis was externalized and removed from the tunica vaginalis. Then, by using a scalpel blade, the head of the epididymis was micro-dissected away from the testis to expose the external rete testis. Mixed germ cells or SSCs positive for SSEA-1 or SSEA-4 suspended in 50 - 100 μ l DMEM/F12 + 4 μ L Trypan Blue (for visualization during the injection), were loaded into a 1-mL syringe and injected into the external rete using a 28G gauge needle (Fig 2.2). Two replicates for each cell type were completed for both age groups, and one five-month-old control was given an injection of DMEM/F12 with only Trypan Blue added.

Figure 2.2. Transplantation of donor cells into recipient cat testes. A cell suspension is injected at the external rete testis under the head of the epididymis (A). Trypan Blue in the suspension allows visualization in the testis after injection (B).



Survival and colonization of SSCs and mixed germ cells in prepubertal cat seminiferous tubules. Prepubertal cat recipients were neutered at 8.5 to 12 weeks post-SSCT. Testes were rinsed with HBSS, epididymides were processed to detect spermatozoa, and seminiferous tubules were analyzed for colony formation and progression through spermatogenesis.

To detect donor sperm, the tunica vaginalis was trimmed away, and the epididymis carefully removed. Epididymides were placed in separate dishes with HBSS, and sperm extracted by pushing the blunt edge of a scalpel blade from the caput to the cauda. Then, epididymides were incised in several locations and incubated for 30 – 45 min to allow sperm remaining within the epididymis to migrate into the medium. The presence of sperm was observed by conventional light microscopy (Olympus IX71, inverted), and samples of sperm were analyzed under fluorescence microscopy (Olympus BX60) to visualize the PKH26 fluorescence.

To evaluate colonization of the seminiferous tubules, testes were weighed and bisected longitudinally. One half of the testis was used for enzymatic digestion and immediate evaluation of donor cell colonization, and the other half was snap-frozen for examination of cross-sections. For enzymatic digestion, the tunica albuginea was removed and seminiferous tubules were minced, transferred to a 15 mL conical tube containing 4 mL Collagenase IV (1 mg/mL; Gibco, Frederick, MD, USA), and incubated in a 38°C water bath for 30 min or until dissociation was complete. Dissociated seminiferous tubules were analyzed under fluorescence microscopy (Olympus IX71), and digital photographs were captured (Olympus DP71).

To snap-freeze, hemi-testis was placed in a tissue mold (VWR, Radnor, PA, USA) filled with OCT (VWR, Radnor, PA, USA). Under a chemical hood, the tissue was submersed in a metal beaker containing a slush of isopentane (VWR, Radnor, PA, USA) chilled by liquid nitrogen. Frozen samples were stored at -80°C or on dry ice before cryosectioning. Slide cryosections were thawed at room temperature, and the presence of red PKH26 fluorescence was observed microscopically (Olympus BX60). Then, tissue sections were stained with DAPI for 1 min, followed by gentle rinsing with PBS and re-examination and digital images were taken (Olympus DP72).

Results

Mixed germ cell isolation. A total of 8 to 12 testes were required to collect a minimum of 10×10^7 mixed germ cells for transplantation of SSCs positive for the pluripotent markers SSEA-1 and SSEA-4. The total mean numbers of mixed germ cells isolated was $1.3 \times 10^8 \pm 5.7 \times 10^7$ cells, and when samples containing $\leq 10 \times 10^7$ cells were sorted, 2% and 1% of live cells were positive for SSEA-1 and SSEA-4, respectively (Fig 2.3, 2.4). In contrast, only two testes were needed to collect an adequate number of mixed germ cells for transplantation if sorting was not done.

Figure 2.3. Forward Scatter vs Side Scatter dot plots for sorting cells stained for SSEA-1 (A) or SSEA-4 (B) antibodies. Cells falling within the polygon gate were sorted as positive (green) or negative (red) for the tested marker. Black dots indicate cells that were dead or heavily granular.

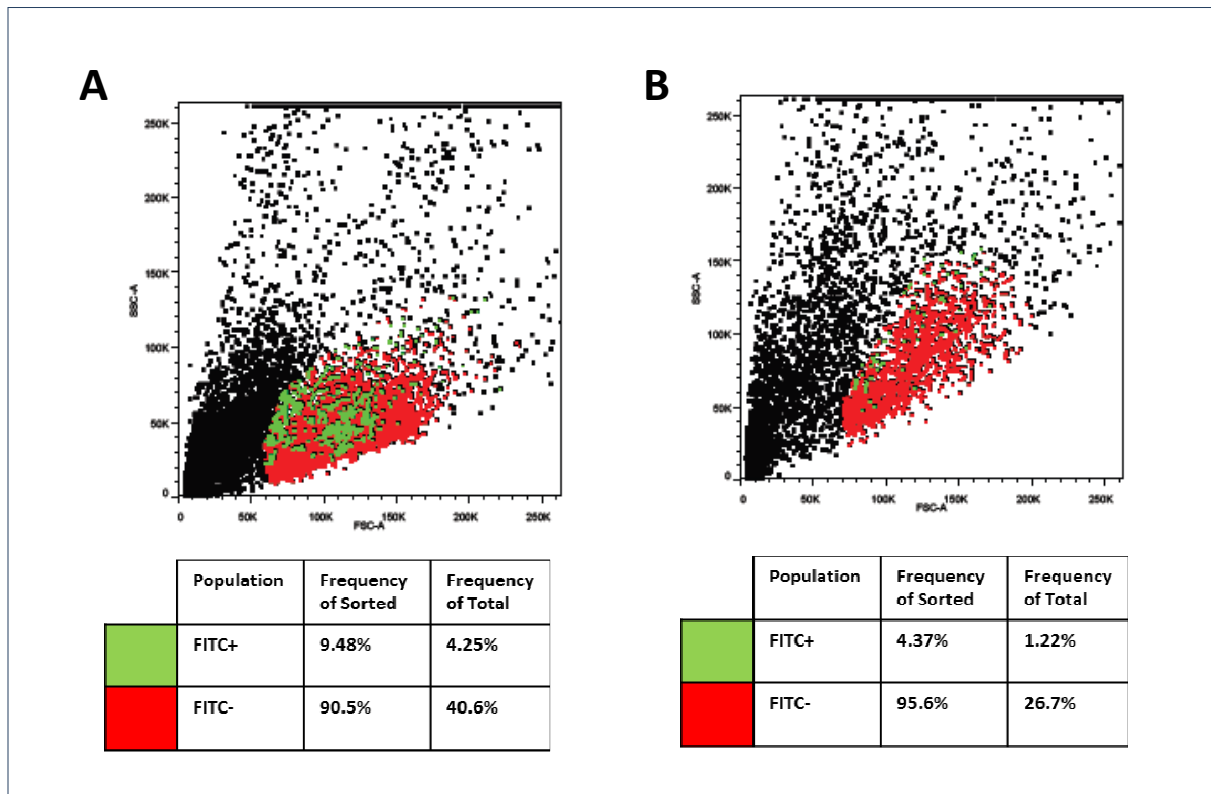
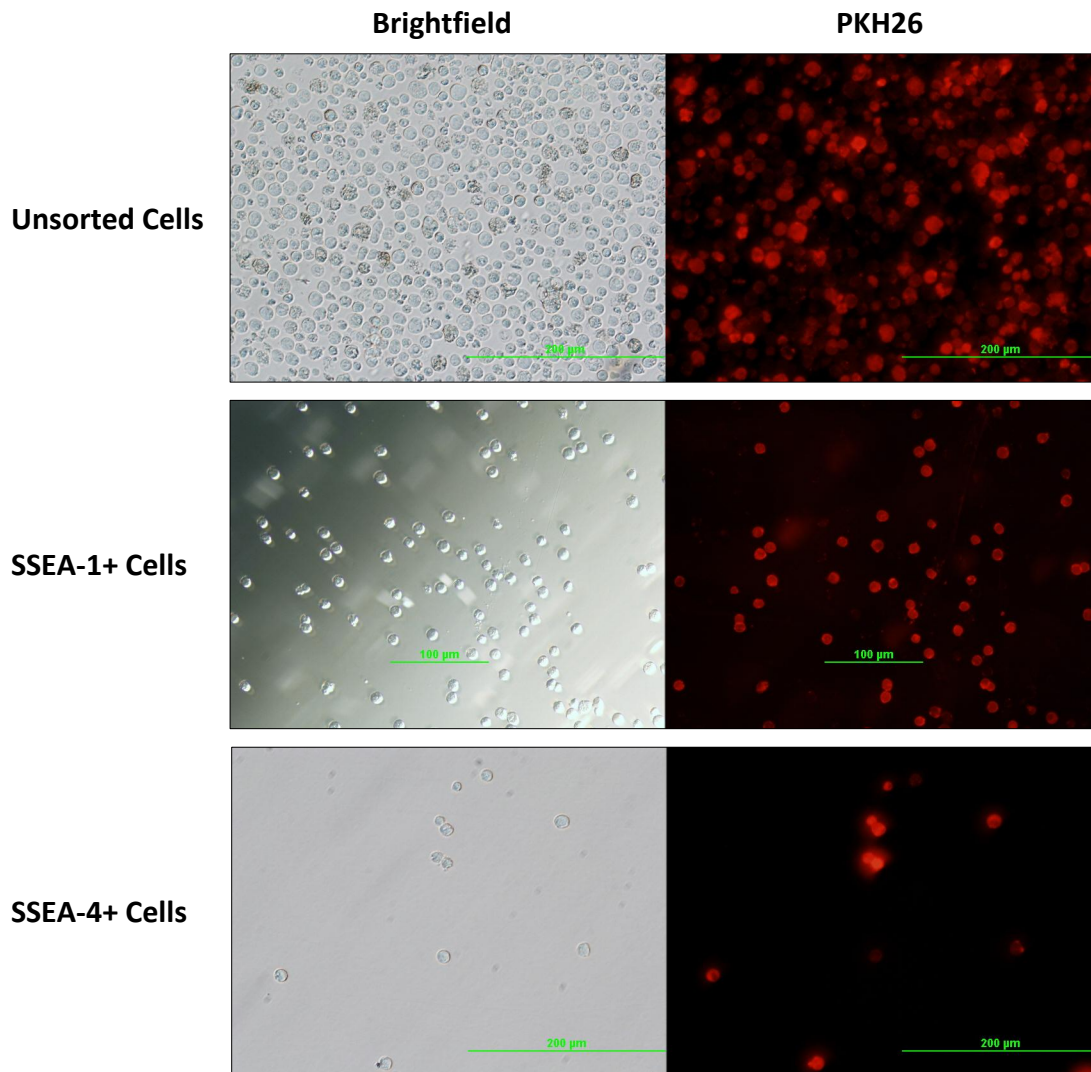


Figure 2.4. Mixed germ cells (unsorted cells) and cells sorted for SSEA-1 or SSEA-4 labeled with PKH26 membrane dye.



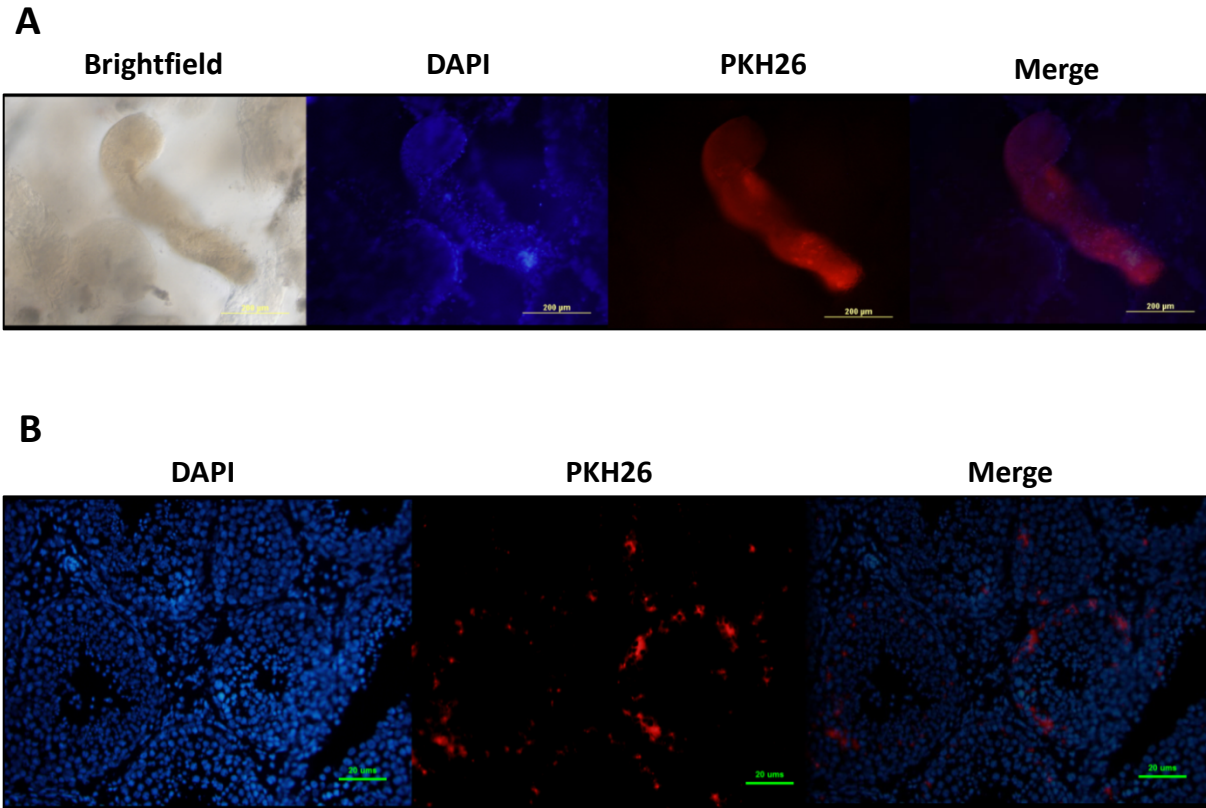
Survival and colonization of enriched SSCs and mixed germ cells in prepubertal cat seminiferous tubules. To evaluate if SSCs sorted as positive for SSEA-1, SSEA-4 or mixed germ cells (unsorted) could colonize successfully in prepubertal male cat recipients without depletion of endogenous germ cells, an average of 250,000 sorted or 10×10^5 unsorted cells were transplanted to each testis (Table 2.1). At 8-12 weeks after SSCT, the testicular weights of recipient cats, with the exception of one, ranged from 0.8 g to 1.25 g, which is considered to be a normal size for seven- to nine-month-old male cats [14].

Tissue evaluation of epididymis and seminiferous tubules showed that, regardless of age or cell type, transplanted cells survived and colonized the seminiferous tubules of prepubertal cats. Five cats (5/12; 42%) had donor cell colonies within the seminiferous tubules, and cats receiving cells sorted for SSEA-4 (2/4; 50%) or SSEA-1 (1/4; 25%) and unsorted cells (2/4; 50%) were localized in small areas at the basal membrane of the seminiferous tubules (Fig 2.5). Colonization was observed in both testes of one five-month-old cat that received cells sorted for SSEA-4+ and in one six-month-old recipient that was transplanted with unsorted mixed germ cells (Table 2.1). Spermatozoa were present in the epididymides of 10 recipients (83%) and in both testes of the control cat receiving injection medium only (Table 2.1); however, PKH26 fluorescence was not observed in any of the sperm samples

Table 2.1. Description of prepubertal male cat recipient's age at SSCs transplantation and neuter. Total of sorted SSCs for SSEA-4 and SSCA-1 and unsorted mixed germ cells transplants, testis weight, presence of spermatozoa and PKH26 red fluorescence of Left/Right testes after neuter.

Recipient ID	Age (m)	Cell Type (injected cells per testis)	Age at Neuter	Testes: Left/Right		
				Weight (g)	Sperm	PKH26
1215	5.5	SSEA-1 (400,000)	7.2	0.89/0.82	Yes, few/Yes, few	No/Yes
1217	6.4	SSEA-1 (200,000)	8.7	1.53/1.41	Yes, motile/Yes, motile	No/No
1247	5.3	SSEA-1 (200,000)	7.8	1.38/1.57	Yes, motile/Yes, motile	No/No
1244	6.3	SSEA-1 (150,000)	8.9	0.86/0.80	None/None	No/No
1224	5.9	SSEA-4 (400,000)	8.6	1.30/1.33	Yes, motile/Yes, motile	No/No
1232	4.9	SSEA-4 (300,000)	7.4	0.68/0.64	None/None	Yes/Yes
1241	5.1	SSEA-4 (100,000)	7.9	1.46/1.46	Yes, motile/Yes, motile	No/No
1251	6.4	SSEA-4 (150,000)	8.9	1.39/1.27	Yes, motile/Yes, motile	No/Yes
1213	5.8	Unsorted (10×10^6)	8.5	1.71/1.58	Yes, motile/Yes, motile	Yes/Yes
1223	6.1	Unsorted (10×10^6)	8.6	1.80/1.69	Yes, motile/Yes, motile	No/Yes
1229	5.1	Unsorted (10×10^6)	7.8	1.48/1.58	Yes, motile/Yes, motile	No/No
1238	5.2	Unsorted (10×10^6)	7.9	0.89/0.92	Yes, motile/Yes, immotile	No/No
1208	4.9	Control (0)	7.4	1.16/1.17	Yes, motile/Yes, motile	-

Figure 2.5. Donor cell colonies labeled with PKH26 red fluorescent membrane dye were detected in enzymatically digested tubules (A) and cross-sections (B) of the testes of some recipient cats. Nuclei were stained with DAPI.



Discussion

Colonization efficiency of SSCs into recipient testis is enhanced by transplantation of subpopulations purified with markers specific for SSCs [17, 27], and by the microenvironment into which they are exposed, when transplanted into immature testes [17, 30]. Previously we had demonstrated that pluripotent surface markers SSEA-1+ and SSEA-4+ are specifically expressed by feline SSCs [78, 79]; however, it is not clear whether purified subpopulations of cat SSCs transplanted into sexually immature cats may improve colonization efficiency and spermatogenesis, resulting in more colonies and an increase in donor sperm production. Therefore, we compared the colonization efficiency after transplantation of subpopulations of cat SSCs purified for SSEA-1+ and SSEA-4+ with that of mixed germ cells. Also, we evaluated the

use of sexually immature domestic cats without depletion of endogenous germ cells as SCC recipients.

Previous studies have identified SSEA-4 as a specific marker for SSCs. For instance, in human and non-human primates, it was shown that all SSEA-4 positive cells are located at the basement membrane of the seminiferous tubules [20, 32, 74, 80], and after xenogenic transplantation of human and non-human primate SSCs into busulfan-treated recipient mouse testes, SSEA-4 positive cells colonized the tubules [20, 74]. Likewise, in the present study, we observed that low numbers of sorted SSCs for SSEA-4+ and SSEA-1+ colonized the seminiferous tubules of sexually immature cat recipient testes. Even though the numbers of transplanted sorted cells were much lower than the number of unsorted cells (mix germ cell population), similar colonization between unsorted and sorted cells was observed. Although our sample size was small, we confirmed that SSEA-4+ and SSEA-1+ germ cell populations contain SSCs and that injections of mixed germ cells were as effective as those of sorted cells in colonizing recipient testes. Nonetheless, the successful colonization by sorted SSCs suggests that SSEA-4 and SSEA-1 are ideal surface markers for purifying subpopulations of SSCs from mixed germ cell populations in the cat.

The methods of chemical injection or radiation exposure by radiography are commonly employed for depletion of recipient endogenous germ cells; however, both present potential risks for inducing cell damage. In domestic cats, both focal irradiation applied to the testes and treatment with busulfan, a cytostatic chemical used for inhibiting cell growth and replication of tumors, have been performed [9, 13]. At the lower dosages of busulfan, depletion of endogenous germ cells was reduced (ranging from 0.6% to 70% of affected seminiferous tubules), while the higher doses of busulfan (single doses of ≥ 25 mg or two doses of ≥ 15 mg) became lethal for the cats [13]. In contrast, focal irradiation was less harmful to the animals and more efficient at eradicating endogenous germ cells ($\sim 97\%$); nonetheless, effects were temporary, and spermatogenesis was regained at 13 weeks post-treatment [9, 13]. To determine if endogenous germ cell eradication improved colonization efficiency, a study comparing busulfan treated adult recipient mice to untreated recipients of the same age found that while donor cell spermatogenesis started earlier in untreated recipients fewer donor

colonies were detected than in busulfan treated recipients [27]. Similarly, in the present study, we observed that spermatogenesis in untreated domestic cats started at 8 to 12 weeks after SSCT. In addition, we determined that some transplanted donor cells were able to establish a niche within the testes of sexually immature cats, despite the presence of the recipient's endogenous germ cells. Therefore, we concluded that depleting endogenous germ cells in the prepubertal domestic cat is not required for successful colonization of donor SSCs; however, a comparison to immature recipients treated for endogenous cell elimination would be necessary to elucidate the efficiency of colonization in untreated immature males.

In a previous study, Tsutsui et al. [14] evaluated spermatogenesis in the domestic cat at different ages. They found that < 25% of the cats had sperm in the testis at five months of age, while at 65% of the six-month-old cats had sperm in the testis and ~ 47% had sperm in the epididymis. By eight months, both testicular and epididymal sperm were observed in 100% of the cats [14]. Since our objective in selecting recipients was to choose cats with fewer endogenous germ cells with which donor cells would compete, yet ensure that sexual hormones for spermatogenesis were established for possible sperm production, we selected five- and six-month-old cats as recipients. Even though we observed colonization of SSCs transplanted into the testes of five- to six-month-old sexually immature male cats, we cannot conclude that colonization efficiency might be related to the absence of endogenous germ cells, since colonization was observed in both six-month-old cats with spermatogenesis likely underway, and five-month-old cats with fewer endogenous germ cells localized in the niche.

It is not clear why sexually immature males do not require elimination of endogenous germ cells for successful colonization and differentiation of transplanted SSCs, but previous studies indicated that the microenvironment of the immature testis enhances SSCs colonization [17, 42]. The authors compared the accessibility of stem cell niches in immature and adult recipients from a mutant mouse strain lacking spermatogenesis and showed that the microenvironment of the immature testis allowed for more efficient fertility restoration than the adult testis [17, 42], with one study reporting 9.4 times more colonization events and colonies that were 4.0 times larger in immature testes than in mature testes [17]. These data suggest that the greater colonization of SSCs into immature testes may be because

spermatogenesis and SSC self-renewal is dominated by factors secreted at high levels in the immature testes. In addition, a more recent study showed that large populations of transit-amplifying mice spermatogonia, or actively dividing spermatogonia fated to differentiate, are found in stages of the seminiferous epithelium with the highest levels of glial cell line-derived neurotrophic factor (GDNF), a product of Sertoli cells signaling SSC self-renewal [86]. Therefore, since cat recipients in our study were in the initial stages of spermatogenesis, we suggest that the ability of colonization in immature cat testes may be in part due to the beneficial microenvironment, and colony expansion may have been restricted to the few tubule segments in stages where both high levels of GDNF and an available niche were present, accounting for the low number of colonies detected. Possibly with an extended period between transplantation and castration, the number of expanded donor colonies could increase.

After SSCT, donor cells must be distinguished from recipient cells. Therefore, in the present study, we used PKH26, a lipophilic membrane dye, which has been used successfully to identify transplanted cells in cats [13], cattle [39], pigs [28], and fish [24] post-SSCT, with fluorescence lasting up to six months [39]. We retrieved cat recipient testes at 8.5 - 12 weeks post-SSCT, allowing sufficient time for colonization (~1 wk [87]) and a full spermatogenic cycle of ~47 days [16], while remaining within the 100 day time frame for PKH26 stability. At castration, the weights of the testes of all recipients except one, fell within or above the expected range for seven- to nine-month-old cats, 0.8 g to 1.25 g, respectively [14], indicating an increase in testicular mass from the normal range of 0.45 g to 0.65 g at the transplantation age of five to six months, respectively. This increase of weight was possibly due to the onset of spermatogenesis, producing numerous mitotic and meiotic germ cells progressing to sperm. The presence of sperm in the epididymis of most testes revealed that full spermatogenesis was established. After completion of donor cell injections, recipient testes were not able to be re-encapsulated in the tunica vaginalis due to swelling caused by the manipulation during externalization, but no serious adverse reactions to the procedure were noted in recipients. Upon castration, minor adhesions to the scrotal skin were seen in half of the recipients, but no obvious developmental defects were discerned except for a “lumpy” appearance in one

recipient in which spermatogenesis appeared to be delayed. Therefore, physical trauma from the transplantation procedure, the presence of donor cells, and the injection medium did not prevent the basic biological processes in the testis. Even though we did not observe PKH26 labeled sperm collected from the epididymides, colonies of donor cells were identified within the seminiferous tubules in five of twelve recipients (41.7%), suggesting that using sexually immature cats may be a viable alternative to chemical or radiation treatment.

Since we did not observe any sperm labeled for PKH26, we performed additional genotyping tests to determine the origin of the epididymal sperm. DNA was extracted from sperm and blood of male recipients (data not shown) and feline microsatellite loci were amplified as previously reported by Gomez et al. [88] using standard ABI fluorescence chemistries on an ABI 377 and sent for analysis. We were not able to identify DNA other than that of the recipient male. Even though we did not observe a different set of DNA loci, we did not have DNA from the donor cells with which to compare because donor cells were pooled from testes of multiple males, to cross analyze the results with recipient DNA. Additionally, it is possible that the number of sperm derived from the donor SSCs may be below the detection level of this method. The low number of donor sperm could be due to several reasons, including the already low number of SSCs available per testis (0.01-0.03% or 1-3 per 10^4 total testis cells in mice [87, 89, 90]), the low colonization rate of transplanted SSCs (~5-12% in mice [87, 90-92]), and the natural degeneration of ~35% of spermatogonia [89]. Furthermore, the overnight incubation in serum-free medium could have affected the colonization ability of SSCs, as reported by Kanatus-Shinohara et al. [90]. Therefore, we could not confirm whether or not some sperm from transplanted donor cells were present, and future studies are needed to identify a reliable method for detection of low numbers of donor sperm in the presence of endogenous germ cells. Transplanting cells pooled from both testes of a single donor, possibly after expansion in an *in vitro* culture system, may result in detectable levels of donor DNA in sperm in addition to allowing for cross analysis of donor DNA with the recipient DNA. Additionally, the production of donor sperm in recipient testes may increase over time. Therefore, allowing more time between transplantation and sperm collection may also result in detectable levels of donor DNA in sperm.

In summary, we found that sexually immature males at, or just prior to the initial onset of puberty were adequate recipients for SSCT. Using five- to six month-old males did not require elimination of endogenous germ cells, thus avoiding the need for chemical or radiation treatments. Donor cells were able to establish a niche within the testes despite the presence of endogenous germ cells, and the transplantation procedures did not inhibit spermatogenesis. Colonization of mixed germ cells confirmed that sorting was not necessary; however, it was revealed that SSCs were present in the SSEA-1+ and SSEA-4+ subpopulations as indicated by the establishment of colonies. The efficiency of colonization of sorted cell populations compared to that of mixed germ cells and in untreated recipients compared to that in endogenous germ cell depleted immature recipients remains to be determined.

CHAPTER 3

DEVELOPING A DOMESTIC CAT SPERMATOGONIAL STEM CELL CULTURE

Abstract

Spermatogonial stem cells (SSCs) and undifferentiated spermatogonia are the only stages of the male gamete capable of replicating, therefore, *in vitro* expansion of these valuable cells would be beneficial to reproductive studies. Although SSCs in other species have been successfully cultured [32, 71, 93, 94], little is known about the *in vitro* requirements of feline SSCs. In this study we evaluated the effect of the presence of serum in the culture medium on the ability of feline mixed germ cells to proliferate *in vitro*. To further enhance proliferation, we sorted cells using SSC-specific markers and evaluated if culturing these cells on inactivated Sertoli cells as a feeder cell layer or supplementing the tissue culture medium with a combination of several growth factors – GDNF, bFGF, EGF, and LIF – and B-27 supplement minus vitamin A could prolong felid SSC self-renewal. We observed that felid mixed germ cells cultured in the presence of serum – 5% fetal bovine serum (FBS) + 15% Knockout Serum Replacement (KOSR) – proliferate during the first week of culture at similar rates to cells cultured in serum-free medium (20% KOSR); however, neither the feeder cells nor supplementation of culture medium with different growth factors or B-27 supplement enhanced SSC proliferation. Cells in these culture conditions were maintained for 5 to 14 days in culture. Further work is needed to identify the factors and other conditions necessary for long-term maintenance and proliferation of domestic cat SSCs.

Introduction

Spermatogonial stem cells (SSCs) have been isolated in fish and numerous mammalian species for transplantation and culture. SSCs comprise only a small percent of the total germ cells in the mammalian testis [90], therefore, the ability to manipulate these cells *in vitro* would not only provide a better understanding of male germ cell biology, but also offer an unlimited supply of these cells for assisted reproduction applications to help propagate genetically

important males. Culturing of fish [71, 95-98], mouse [19, 94, 99], and human SSCs [30, 32] has been extensively studied; however, little has been reported regarding cat SSC culture [73].

To allow long term maintenance and proliferation of the stem cell-like undifferentiated spermatogonia *in vitro*, it is necessary to identify the proper conditions and medium components. To this end, SSC culture studies often refer to the requirements for stem cell cultures, which differ by species [100]. However, SSCs reside in a complex environment in which self-renewal and differentiation are regulated by extrinsic factors from the somatic environment and by intrinsic transcription factors expressed by SSCs, making the identification of some of the essential factors difficult. Various combinations of media and supplements have been tested for culture of SSCs from different species, further revealing that the requirements of SSCs also vary among species [42, 46, 101-103].

Traditionally, embryonic stem cells (ESCs) are cultured over a mitotically inactivated fibroblast feeder layer [60, 72, 99, 104, 105], which many SSC cultures have also employed [72, 91, 106]. However, some SSC studies have utilized feeder layers consisting of inactivated Sertoli cells [19, 73, 107], the somatic cells in testis that govern the process of spermatogenesis, while others have sought to maintain SSCs in a feeder-free culture in an effort to more clearly identify the factors necessary for proliferation [90, 108]. There has also been experimentation with media lacking fetal bovine serum (FBS), which contains growth promoting factors and other components required in cellular metabolism due to concerns about cross-species contamination, unknown constituents in serum, and the presence of factors that may promote differentiation [90, 99]. The unknown components in FBS also make the effects of other added factors difficult to analyze. To eliminate these concerns, serum replacement supplements have been tested [105], and more recently, B-27 supplement without vitamin A has been evaluated [103, 104].

In addition to protein supplements, the influence of different growth factors on colony formation has been assessed [108]. The primary factor added to SSCs in culture is glial cell line-derived neurotrophic factor (GDNF) which is secreted by Sertoli cells and plays an important role in SSC self-renewal [72, 105]. Several studies have compared the effects of GDNF in combination with other factors frequently used in ESC culture [53, 60, 100] including leukemia

inhibitory factor (LIF), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF) [90, 93, 104].

The purpose of the present study was to determine if domestic cat mixed germ cells and subpopulations of purified cat SSCs can proliferate *in vitro*. Specifically, we evaluated the necessity of serum for SSC proliferation, as well as the effect of Sertoli cell co-culture and supplementing medium with combinations of growth factors and B-27 supplement minus vitamin A on SSC self-renewal.

Materials and Methods

Chemicals. All chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

Tissue handling and germ cell isolation. Adult testes (n=31) obtained from routine castration were provided by local veterinary clinics and transported to the lab in HEPES buffered saline solution (Ultrasaline A Solution, Bio-Whittaker, Walkersville, MD, USA) where they were either processed immediately or stored \leq two days at 4°C.

Testes were rinsed in Hanks' Balanced Salt Solution (HBSS), and external tissue including the epididymis and tunica albuginea were carefully removed to expose the seminiferous tubules. Tubule tissue was incubated for 30-45 min in HBSS supplemented with 25 μ g/mL amphotericin-B, 10 μ g/mL streptomycin, and 50 μ g/mL gentamicin, and mixed testicular cell suspensions were obtained using a two-step enzymatic digestion, as previously described [9], with modifications. After the final enzymatic incubation, dual filtration through 100 μ m and 40 μ m nylon mesh filters (BD Falcon, Tewksbury, MA, USA) removed large cells and cell aggregates. Filtered cell suspensions were further separated by Percoll density gradient centrifugation. Briefly, decreasing concentrations of Percoll (35.0%, 27.5%, and 20.0%) diluted with HBBS were layered in a 15 mL tube. Then, filtered cell suspensions were added slowly to the top layer of the Percoll gradient, and separation was achieved by centrifugation at 725 x g for 25 min. Cell suspensions containing spermatogonial cells formed bands at the tops of the 27.5% and 35.0% Percoll layers and were pooled and plated for differential cell adhesion culture.

SSC *in vitro* culture. Spermatogonial cells were plated in 35-mm tissue culture containing 2 mL stem cell medium (DMEM/F12; Gibco, Frederick, MD, USA) supplemented with 5% fetal bovine serum (FBS; Hyclone/GE Healthcare Life Sciences, Piscataway, NJ, USA) and 15% Knockout Serum Replacement (KOSR; Invitrogen, Carlsbad, CA, USA) or 20% KOSR (serum free), 1 mM L-glutamine, 0.1 mM β -mercaptoethanol, 1.25% non-essential amino acids, 10 μ L/mL amphotericin-B, 10 μ g/mL streptomycin, and 50 μ g/mL gentamicin and GDNF (10 ng/mL) and cultured at 38.5°C in 5% CO₂/air. Albumin-associated lipids in the KOSR have been found to strongly stimulate undifferentiated hESC proliferation [109], and therefore, was also used in medium with serum to maximize SSC proliferation. Using differential cell adhesion culture, attached somatic cells were able to be separated from germ cells.

Fluorescent activated cell sorting (FACS). To isolate SSCs from a mixed germ cell population, samples were sorted for single stained cells positive for either SSEA-1 or SSEA-4 or double stained cells positive for both SSEA-4 and GFRA1 as reported by Powell et al. [78]. Briefly, mixed germ cells were incubated with primary antibodies-- mouse anti-SSEA-1 (1:50 dilution; Millipore, Billerica, MA, USA), mouse anti-SSEA-4 (1:50 dilution; Millipore, Billerica, MA, USA), or mouse anti-SSEA-4 in combination with rabbit anti-GFRA1 (1:50 dilution; Abcam, Cambridge, MA, USA; Table 1.1). Antibody binding reactions were carried out in a blocking solution of 1% bovine serum albumin (BSA), and 5% goat or 5% sheep serum in DPBS for 45 min on ice. Cells were then washed with DPBS + 1% BSA and incubated to detect SSEA-1 with secondary goat anti-mouse IgM Alexa Fluor 488 conjugated (1:100 dilution; Invitrogen/Life Technologies, Carlsbad, CA, USA), SSEA-4 with secondary sheep anti-mouse IgG conjugated with fluorescein isothiocyanate-FITC (1:256 dilution; Invitrogen/Life Technologies, Carlsbad, CA, USA), and GFRA1 with goat anti-rabbit Alexa Fluor 647 (1:500; Invitrogen/Life Technologies, Carlsbad, CA, USA). After incubation, cells were washed with DPBS + 1% BSA, and pellets were re-suspended in DMEM/F12 medium (to a concentration of 10×10^6 cells/mL) and kept on ice until sorting. Controls for nonspecific auto-fluorescence were performed by replacing the primary antibody with goat or sheep IgG and cultured with secondary antibody. Unstained cells were also used as controls. Cells were sorted using one of two flow cytometers (FACSCalibur

and FACSAria, Becton Dickinson Biosciences, San Jose, CA, USA) with parameters set using the software FACSDiva (Becton Dickinson Biosciences, San Jose, CA, USA).

Preparation of Sertoli feeder cell layers. Fresh mixed germ cells were plated in a T-75 culture flask for one week. Medium containing floating cells was removed and an equal volume of stem cell medium supplemented with serum was added to the remaining plated somatic cells, which included Sertoli cells. Upon reaching 90% confluence within 4 to 5 days, Sertoli cells were inactivated by incubation with 40 µg/mL of mitomycin-C for 4 to 5 h. After inactivation, Sertoli cells were dissociated with 2.5 mg/mL of trypsin, washed 2X in DPBS and plated in gelatin-coated 12-well dishes (Nunc, Roskilde, Denmark) at a density of 10×10^4 cells/well. To differentiate inactivated Sertoli cells from newly plated spermatogonial cells, Sertoli cell membranes were labeled with the PKH26 Red Fluorescent Cell Linker Mini Kit, following manufacturer's guidelines with minor modifications. Briefly, stem cell medium was removed, and each well gently washed with DPBS. Cells were then incubated in a dye solution of 250 µL Diluent C (PKH26 Fluorescent Cell Linker Kit) + 0.5 µL PKH26 for 5 min at room temperature. To bind the excess dye, an equal volume of DMEM/F12 medium was added for 1 min. Each well was washed with DPBS, and 0.5 mL of stem cell medium/well was added before plating mixed germ cells or sorted SSCs.

Experimental design. *Experiment 1: Effect of serum on the ability of cat mixed germ cells to proliferate in vitro.* Freshly isolated mixed germ cells from a single testis were equally divided and plated in stem cell medium supplemented with either (1) serum or (2) serum-free medium. Half of the stem cell medium was changed every two days and replaced with an equal volume of fresh medium.

Experiment 2: Cell growth of SSCs cultured on Sertoli cell feeder layers and stem cell medium supplemented with bFGF. SSCs purified for SSEA-4+ were plated on mitotically inactivated Sertoli cell feeder layers in 12-well dishes containing 0.5 mL of stem cell medium/well supplemented either with (1) serum or (2) serum-free medium and (3) GDNF (20 ng/mL or (4) GDNF + bFGF (25 ng/mL; Invitrogen/Life Technologies, Carlsbad, CA, USA). Half of the medium was replaced with fresh medium and factors every two to three days. Cells were

visualized in bright field and under fluorescence using an Olympus IX71 microscope. Images were taken with an Olympus DP71 digital camera.

Experiment 3: Effect of supplementation of stem cell medium with various combinations of growth factors and B-27 supplement on SSC proliferation. To evaluate the effect of growth factors, SSCs purified for (1) SSEA-1+, (2) SSEA-4+, or (3) mixed germ cells were plated in a 12-well dish with 1 mL of serum-free stem cell medium/well supplemented with various combinations of growth factors: (4) GDNF (10 ng/mL), (5) GDNF + bFGF (20 ng/mL), (6) GDNF + bFGF + EGF (20 ng/mL), or (7) GDNF + bFGF + EGF + LIF (20 ng/mL; not added until day 2 of culture). Every two to three days, half of the medium was replaced with fresh medium and growth factors.

To evaluate the effect of B-27 supplement on the stem cell medium supplemented with various combinations of growth factors, SSCs dual-stained and purified for (1) SSEA-4+, GFRA1-, (2) for SSEA-4+, GFRA1+, or (3) mixed germ cells were plated in a 24-well dish with 0.5 mL of serum-free stem cell medium supplemented with B-27 minus vitamin A (1X; Invitrogen/Life Technologies, Carlsbad, CA, USA). Stem cell medium was supplemented with the same combination of growth factors as described above. Half the medium was replaced with fresh medium and growth factors every two to three days.

Cell growth in all experiments was evaluated with an Olympus IX71 microscope, and digital images captured with an Olympus DP71 camera.

Results

Experiment 1: Effect of serum on the ability of cat mixed germ cells to proliferate *in vitro*. As shown in Figure 3.1, the presence of serum in the stem cell medium did not affect the ability of SSCs to proliferate *in vitro*. In fact, spermatogonia were maintained in culture in a serum-free or serum supplemented medium for up to 14 days. However, the cellular growth pattern of spermatogonia cells cultured with serum was different than that found in spermatogonia cells cultured in serum-free medium. In the presence of serum, grape-like clusters of spermatogonia were observed between days 2 and 8, but few spermatogonia were visually identifiable by day 14. Moreover, somatic testicular cells (including Sertoli cells) reached confluence within one week. In contrast, in serum-free medium, grape-like clusters of

spermatogonia were also visible between days 2 and 8, but individual and small clusters of spermatogonia were still present between days 8 and 14, and low numbers of Sertoli cells plated on the dish. Even though fewer numbers of spermatogonia were observed in cells cultured with serum, tightly packed colonies developed in both serum (not shown) and serum-free conditions (Fig 3.1).

Experiment 2: Cell growth of SSEA-4+ SSCs cultured on Sertoli cell feeder layers and stem cell medium supplemented with bFGF. Cell growth of SSEA-4+ SSCs was not affected by either the presence of Sertoli feeder cells or supplementation of the medium with bFGF (Fig 3.2). Nonetheless, SSCs attach onto or near to the somatic cells. In the presence of stem cell medium supplemented with serum, SSEA-4+ SSCs formed small grape-like clusters in which cells could be individually identified, while in serum-free medium, SSCs were difficult to distinguish. However, few SSEA-4+ SSCs were visible after four days in culture, and the feeder layer cells became apoptotic regardless of medium.

Figure 3.1. Comparison of cells cultured with and without FBS supplementation. Mixed germ cells were cultured in SSC medium with either 15% KOSR + 5% FBS (SSC medium with serum) or 20% KOSR (serum-free SSC medium) and were maintained up to two weeks. Somatic cells rapidly plate and replicate in the presence of FBS. 100X magnification.

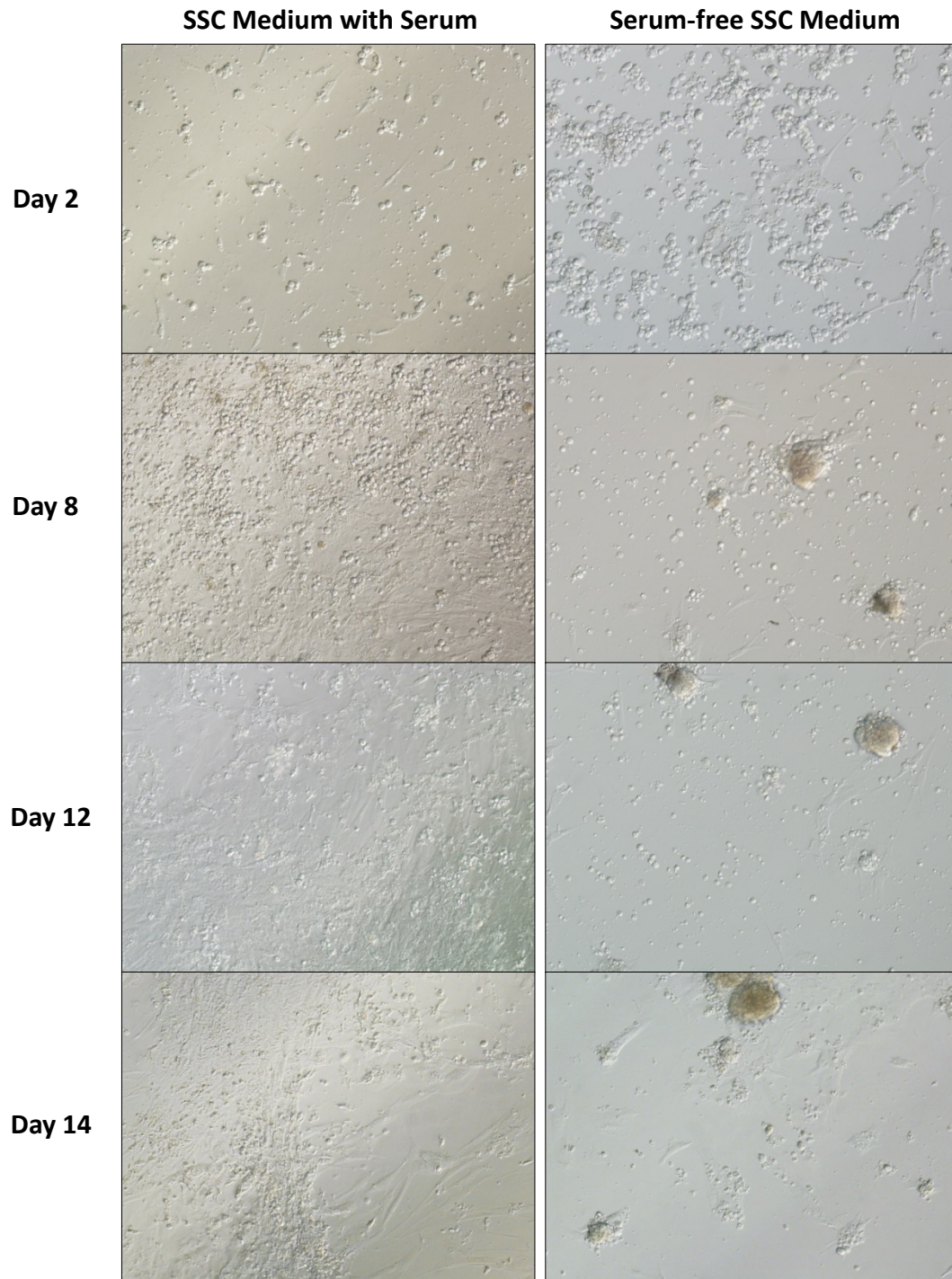
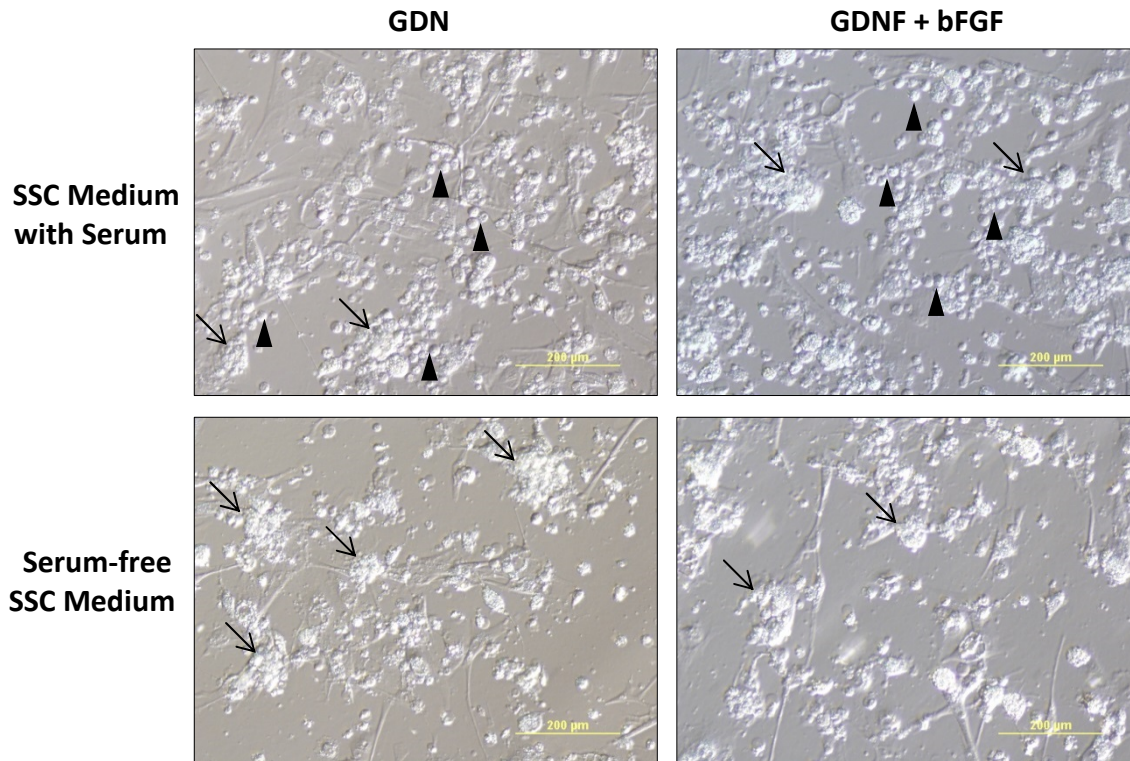


Figure 3.2. SSEA-4+ cells on day 1 of culture with an inactivated Sertoli cell feeder layer. Cells were cultured in media supplemented with GDNF, either with or without serum present, and with or without additional supplementation with bFGF. Grape-like clusters of individual SSCs (arrow heads) formed in the presence of serum, and clusters in which individual SSCs were indistinguishable (arrows) developed under all conditions. 200X magnification



Experiment 3: Effect of supplementation of stem cell medium with various combinations of growth factors and B-27 supplement on cell proliferation of SSCs. Cell proliferation of purified SSCs or mixed germ cells was not enhanced by the combination of growth factors or the presence of B-27 supplement in the stem cell medium as summarized in Table 3.1. Under various combinations of growth factors, we observed that few purified SSCs or mixed germ cells attached by day 2. The few attached cells were single, pairs, and small clusters of spermatogonia (Fig 3.3). By day 5, the number of plated cells increased in all cultures, but the presence of plated somatic cells was greater in the SSEA-4+ SSCs and mixed germ cell cultures. Mostly pairs and clusters of spermatogonia were observed at this time point (Fig 3.4). The somatic cells in the SSEA-4+ SSC cultures that were supplemented with other factors in addition to GDNF reached nearly 100% confluence by day 8, and spermatogonia attached to the

somatic cells to form small clusters or colonies (Fig 3.5). There was little change in somatic cell growth in other culture conditions, and while some individual or small clusters of spermatogonia were observed, mostly large floating clusters of cells had formed.

In medium supplemented with B-27 supplement minus vitamin A, purified SSCs and mixed germ cells were plated by day 3 of culture as pairs and small clusters of spermatogonia and few somatic cells were plated (Fig 3.6). Nonetheless, purified SSCs became apoptotic by day six.

Table 3.1. Effects of different growth factors on SSEA-1+ and SSEA-4+ SSC replication and colony formation (A) or on dual-stained SSEA-4+, GFRA1+ and SSEA-4+, GFRA1- SSC replication and colony formation in the presence of Supplement B27 minus Vitamin A (B).

A.

Factors in SSC Medium								
	GDNF only		GDNF, bFGF		GDNF, bFGF, EGF		GDNF, bFGF, EGF, LIF	
	Rep.	Col. Form	Rep.	Col. Form	Rep.	Col. Form	Rep.	Col. Form
SSEA-1+	+	Cl	+	Cl, A	+	P, Cl, A	+	P, Cl, A
SSEA-4+	+	P, Cl, A	+	P, Ch, Cl, A	+	P, Ch, Cl, A	+	P, Ch, Cl, A
Mixed Germ Cells	+	P, Cl, A	+	P, Cl, A	+	P, Ch, Cl, A	+	P, Ch, Cl, A

B.

Factors in SSC Medium Supplemented with B27								
	GDNF only		GDNF, bFGF		GDNF, EGF		GDNF, LIF	
	Rep.	Col. Form	Rep.	Col. Form	Rep.	Col. Form	Rep.	Col. Form
SSEA-4+, GFRA1+	+	P, Cl, A	+	P, Ch, Cl, A	+	P, Cl, A	+	P, Cl, A
SSEA-4+, GFRA1-	+	P, Ch, A		N/A		N/A		N/A
Mixed Germ Cells	+	P, Cl, A	+	P, Ch, Cl, A	+	P, Cl, A	+	P, Cl, A

Rep, replication; Col. Form, colonies formed; P, pairs; Ch, chains; Cl; clusteres; A, attached.

Figure 3.3. Day 2 of culture of mixed germ cells and purified SSCs for SSEA-1+ and SSEA-4+. Cells were cultured in serum-free (20% KOSR) stem cell medium supplemented with various combinations of growth factors GDNF, bFGF, and EGF. “+” indicates addition of the factors above as well. 200X magnification.

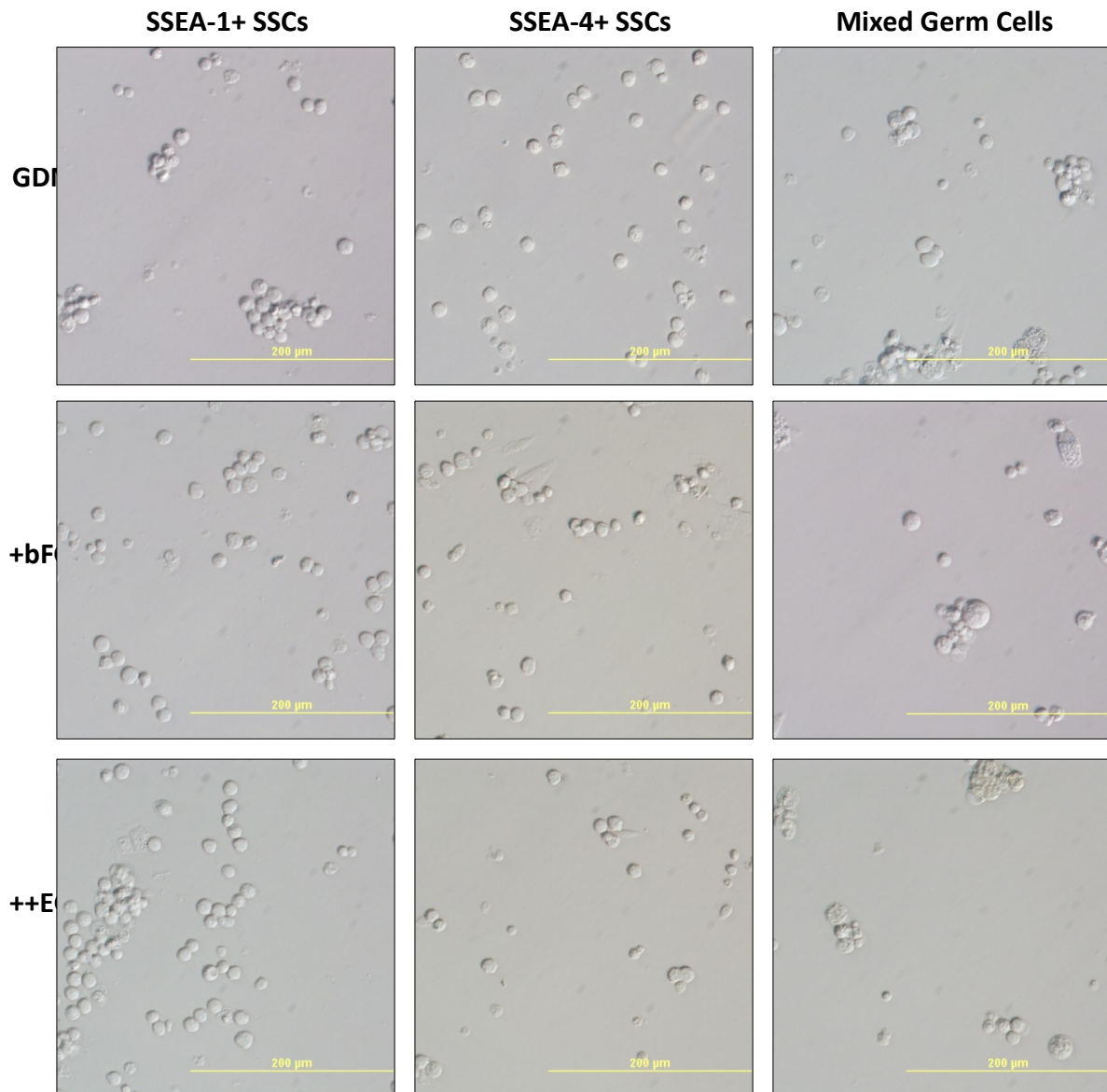


Figure 3.4. Day 5 of culture of mixed germ cells and purified SSCs for SSEA-1+ and SSEA-4+. Cells were cultured in serum-free (20% KOSR) with combinations of factors GDNF, EGF, bFGF, and LIF. “+” indicates addition of the factors above as well. 200X magnification.

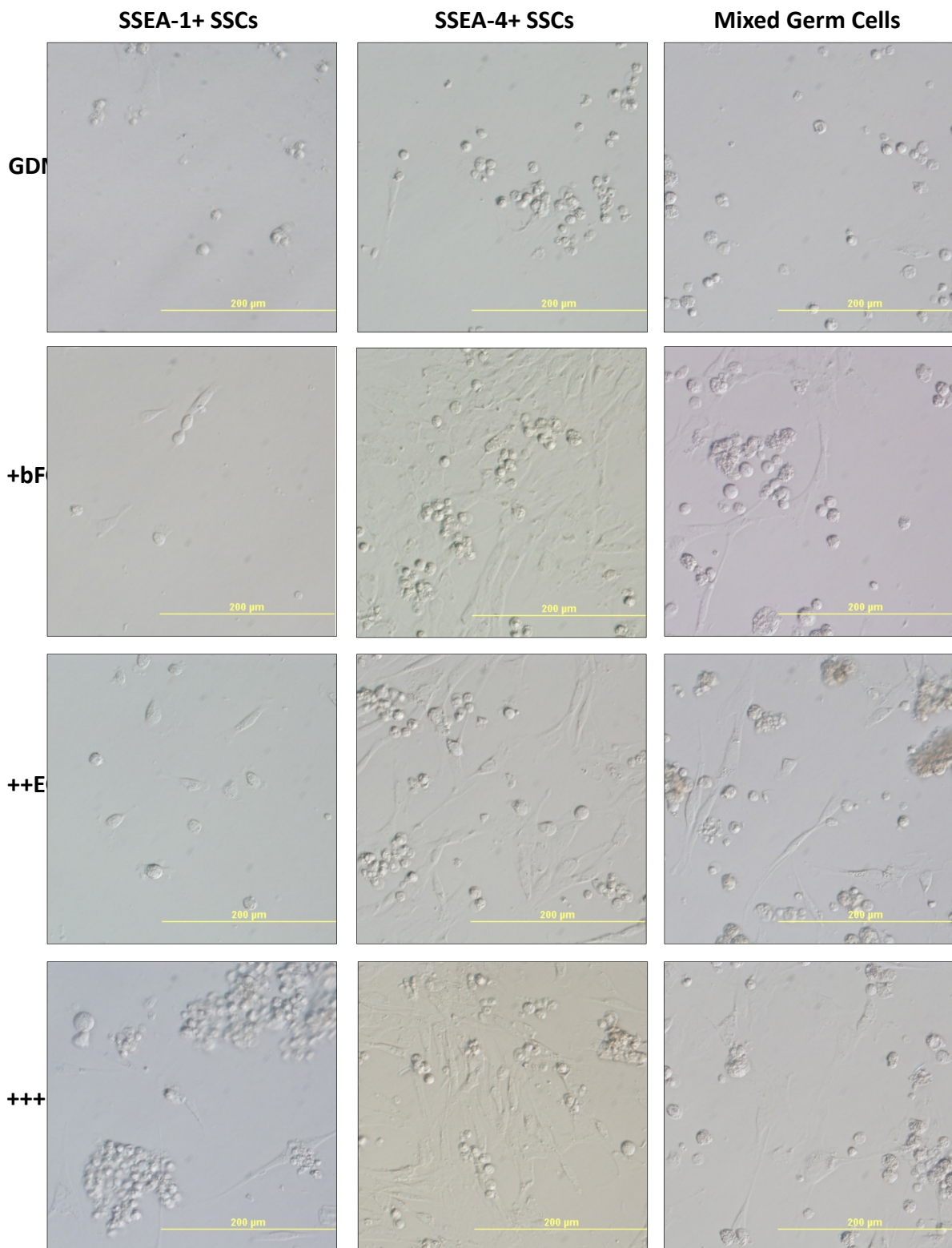


Figure 3.5. Day 8 of culture of mixed germ cells and purified SSCs for SSEA-1+ and SSEA-4+. All cells were cultured with serum-free (20% KOSR) stem cell medium supplemented with combinations of factors GDNF, EGF, bFGF, and LIF. “+” indicates addition of the factors above as well. 200X magnification.

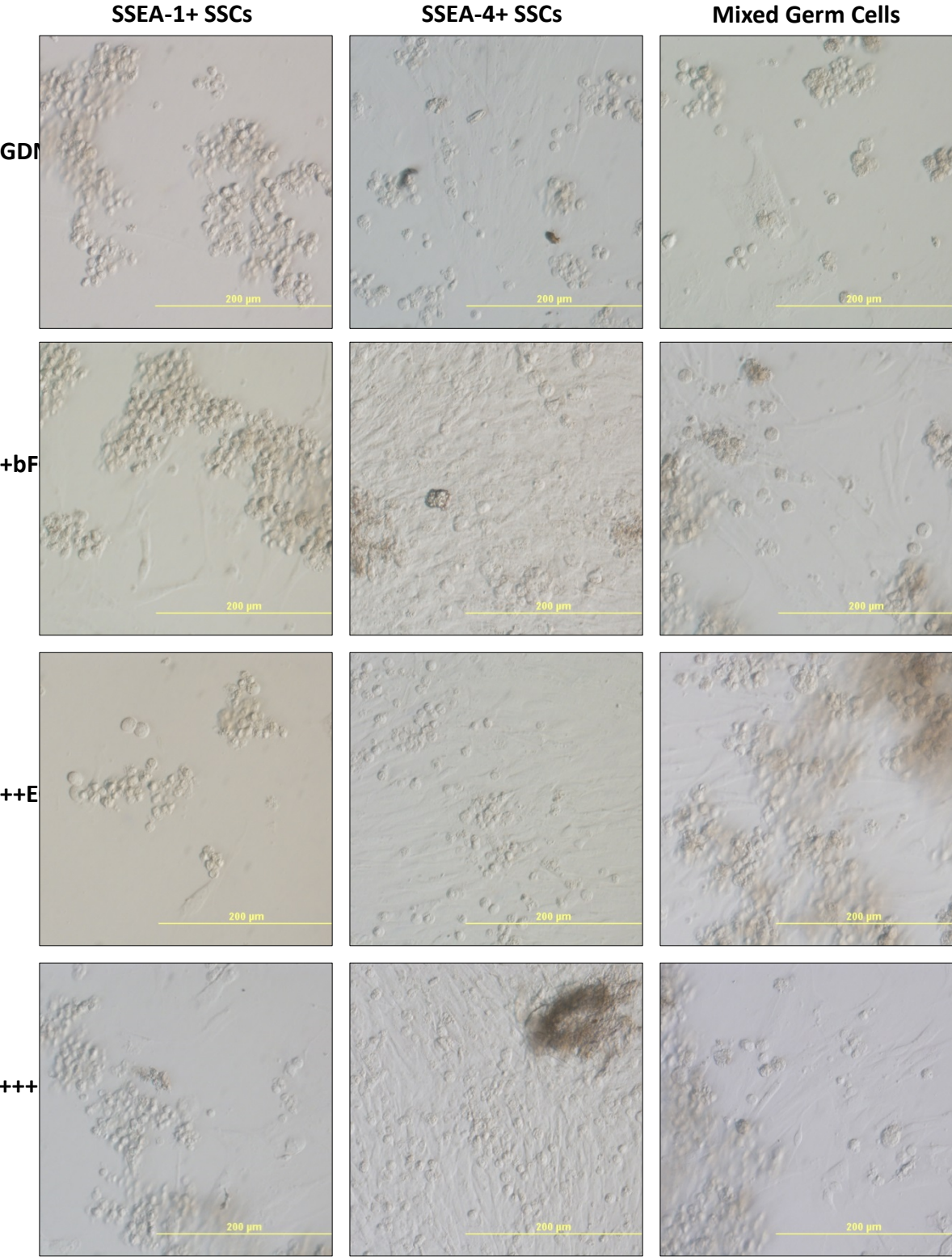
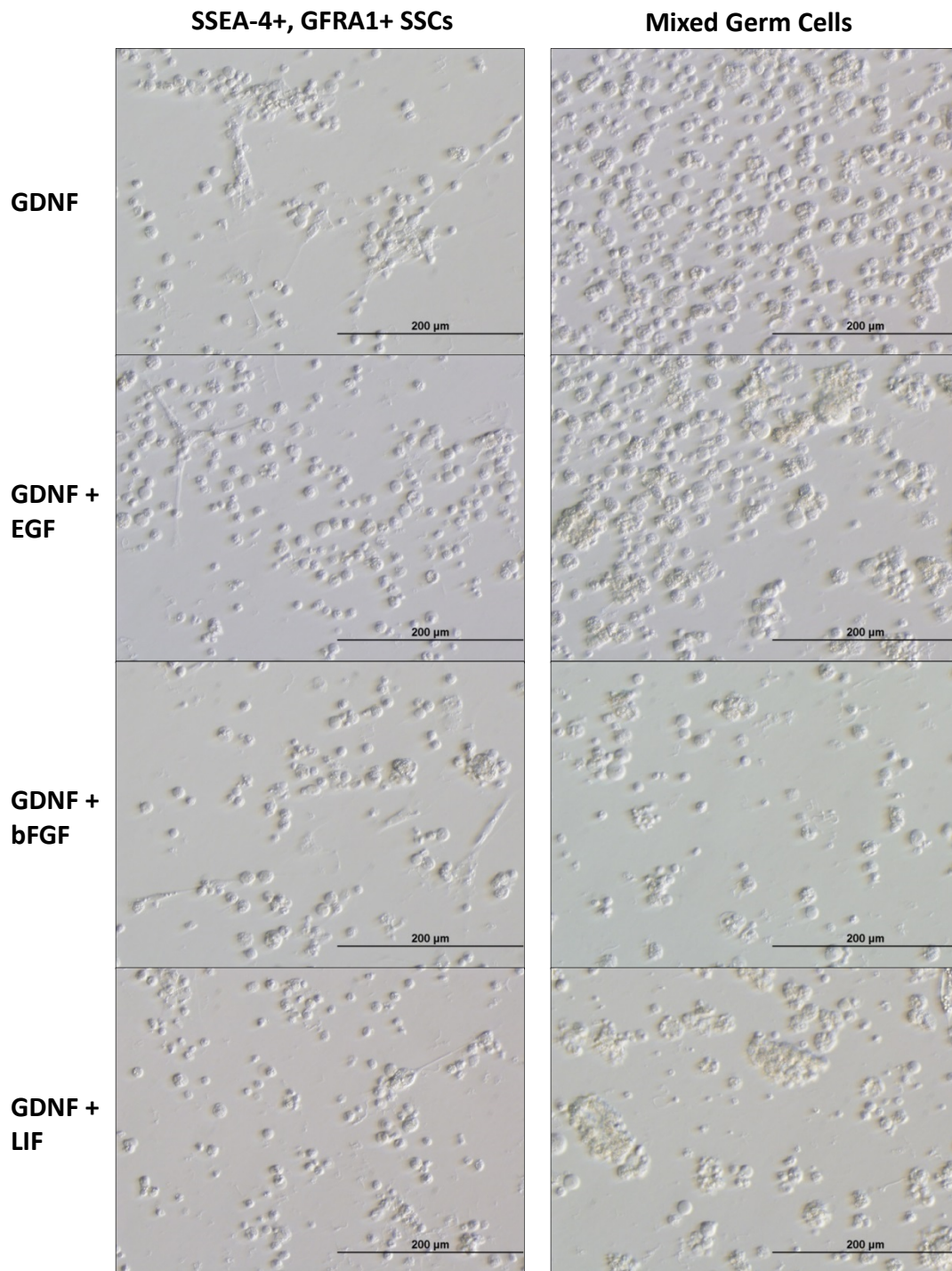


Figure 3.6. Day 3 of SSEA-4+, GFRA1+ cells and unsorted cells in culture supplemented with GDNF only or in addition to EGF, bFGF, or LIF. Cells were cultured in SSC medium with the supplement B-27 minus vitamin A and 20% KOSR. 200X magnification.



Discussion

In this study, we examined several components of SSC culture, including serum, feeder layers, and various growth factors, to elucidate the requirements for cat SSC maintenance and proliferation. We were able to maintain SSCs in the form of pairs, chains, clusters, and defined colonies for up to 14 days in short term culture with stem cell media containing various combinations of supplements; however, functional assays are required to determine possible changes in the SSC molecular characteristics caused by the various media conditions.

Cat SSCs were maintained *in vitro*, at least short-term, regardless of the presence or absence of serum (FBS) in the medium. In addition to chains and clusters of SSCs, well-defined colonies developed in mixed germ cells after one week in culture (Fig 3.1, 3.2), similar in appearance to those identified in bovine [93, 110], canine [111], and murine [112] mixed germ cell cultures and earlier than previously reported in cat SSC culture [73]. However, the spermatogonial cells were sometimes more difficult to visualize in the mixed germ cell cultures grown in stem cell medium containing 5% FBS due to the high number of testicular cells present and the rapidly replicating somatic cells (Sertoli cells) that plated. Few somatic cells were able to attach and replicate in serum-free medium (Fig 3.1), indicating that FBS may provide nutrients necessary for Sertoli cells but not for SSCs.

Although cat SSCs replicated in serum-free medium, it was observed that colonies frequently started on or near plated somatic cells, as reported in other species [32, 93, 108]. Therefore, we investigated the use of a mitotically inactivated Sertoli cell feeder layer to support replication of purified SSCs. In the presence of serum, SSEA-4+ SSCs formed small grape-like clusters over the Sertoli cells, whereas only a few individual and pairs of SSEA-4+ SSCs could be distinguished in the serum-free medium (Fig 3.3). These results clearly indicated that the presence of serum in the culture medium is an important factor for maintaining Sertoli cell replication and consequently support SSC growth. However, even with serum in the culture medium, Sertoli cells became apoptotic with detached cells and floating cell debris by day 4 of culture. The exact reason for this rapid decline is unknown, but it is possible that cat Sertoli cells failed to thrive due to inactivation or passaging. In fact, in previous experiments using

replicating Sertoli cell cultures, we observed that after the second passage, Sertoli cells were not able to attach to the tissue culture dish.

Even though cat inactivated Sertoli cells have been used as feeder cells for co-culture of cat SSCs [73] the authors did not report the plating efficiency or condition of the inactivated cells after several days of culture. More importantly, the two colonies used for co-culture on inactivated Sertoli feeder cells were initially grown under other conditions before passaging to the Sertoli cell feeder layer, and only one colony continued to proliferate for ~3 weeks until it degenerated [73]. Similar to our study, cat SSC could not be maintained on inactivated Sertoli cells. However, in other species, the use of Sertoli cell feeder layers without inactivation, after being plated along with mixed germ cells, supported the growth of SSCs [19, 110, 112]. Thus, it is possible that inactivation may disrupt the ability of cat Sertoli cells to support the proliferation of SSCs.

The use of feeder layers and FBS, however, may increase the difficulty of accurately evaluating other culture factors. In particular, the addition of FBS to culture medium raises several concerns, including the possibility of cross-species contamination and the presence of unknown and/or differentiation inducing components [72, 90, 108]. Several studies have sought to produce well-defined culture conditions for SSCs by eliminating the use of serum, feeder cells, or both and implementing serum replacements and various growth factors [70, 90, 108, 113]. StemPro-34A [30, 32, 73, 90] and KOSR [32, 105] are commonly used supplements for replacing FBS in the culture medium, but, more recently, some studies have used the B-27 supplement without vitamin A [103, 104]. The B-27 supplement contains many of the individual additives that Kanatsu-Shinohara et al. [94] used successfully for serum-free mouse SSC culture, but B-27 is more simplified and cost effective [104]. Supplementation with B-27 did not have an observable effect on cat SSCs (Fig 3.7). It is possible that our serum-free medium, containing KOSR, partially satisfies some of the requirements for cat SSC survival in culture with the addition of GDNF and indicates that additional nutrients in B-27 are not necessary for cat SSCs.

Serum-free culture conditions have been used for the culture of SSCs in several mammalian species with dissimilar results. For example, in humans, spermatogonia cells

positive for GPR125+ were maintained in culture for a short- period of time under serum-free and feeder-free culture conditions [30]. However, Van de wee et al. [19] reported that, although purified mouse spermatogonial cells survived in serum-free media over a Sertoli feeder layer *in vitro* for > 25 days, the cells were not able to replicate [19]. Other studies in bovine and mice found that, without serum, fewer colonies formed [70, 90], and there was a decline in offspring efficiency after transplantation and donor sperm collection in mice [90]. Therefore, these results clearly indicated that SSCs may require certain factors present in serum to develop normally into spermatozoa and produce live healthy offspring. Even though it is not clear which factors are present in FBS, medium supplementation with different growth factors may enhance viability of SSCs.

GDNF is the most widely used factor in SSC cultures due to its role in Sertoli cell-SSC signaling, but other factors, such as bFGF, EGF, and LIF, have also been included in SSC culture media with some degree of success [73, 89, 93, 104]. Likewise, in this study, cat SSCs were able to be maintained in feeder-free and serum-free medium, and in the presence of various growth factors for up to eight days. We observed pairs, chains, and clusters of SSCs plated at similar numbers in the presence of GDNF alone or in combination with other growth factors, clearly indicating that GDNF alone may be sufficient for cat SSC maintenance. Interestingly, some somatic cells within those samples did plate, mostly in the group of purified SSCs positive for SSEA-4 in which GDNF was supplemented with other factors and the fewest plated cells were seen in the group of purified SSCs for SSEA-1+. The presence of somatic cells in the purified SSCs further suggests that only a subpopulation of purified cells was SSCs and that multipotent somatic stromal cells may also be positive for pluripotent surface markers SSEA-1 and SSEA-4 [77]. Similarly, it was reported that human SSCs positive for SSEA-4 also contained somatic cells which plated and supported the further formation of SSC colonies [32]. In dogs, Lee et al. [111] determined that SSC colonies could form in medium containing either a serum replacement or an adequate amount of serum ($\geq 5\%$), in addition to supplementation with both factors GDNF and bFGF. Aponte et al. [93] found that GDNF alone was sufficient to allow for bovine SSC growth, but GDNF combined with bFGF, EGF, and LIF all together enhanced SSC culture by stimulating Sertoli cell growth. Some factors, however, have been found to have a negative

effect on SSCs depending on species [93, 99], such as an inhibitory effect of bFGF and higher concentrations of EGF on mouse SSCs [99]. While the addition of factors other than GDNF may not be required for cat SSCs in culture, further investigation is needed to determine if somatic cell growth in purified cat SSC cultures could be stimulated with additional factors to improve SSC growth, particularly in purified cell populations where few somatic cells are present.

In summary, defining culture conditions for cat SSCs has proven to be difficult, especially for subpopulations enriched in SSCs through cell sorting. Preliminary results have shown that only the mixed germ cell population formed compact, defined colonies, while pairs, chains, and small clusters of spermatogonia formed in purified cultures of SSCs. Furthermore, a serum-free medium supplemented with GDNF was sufficient for short term maintenance of SSCs *in vitro*; however, purified SSCs were not able to be maintained as long as non-purified mixed germ cells. It is possible that a feeder of actively growing Sertoli cells may improve the growth of cat SSCs. Therefore, further investigation into the effects of growth factors on purified cell subpopulations of SSCs and the use of somatic cells as feeder layers to evaluate possible benefits and population-specific biological interactions is necessary.

CHAPTER 4

PHENOTYPIC CHARACTERIZATION AND XENOGENIC TRANSPLANTATION OF LION SPERMATOGONIAL CELLS

Abstract

Recent studies have investigated the manipulation of spermatogonial stem cells (SSCs), the precursors to spermatozoa, for transplantation as an assisted reproduction technique to aid the conservation of endangered species [13, 114]. A related domestic species as a recipient has been desirable as they may be easier to obtain and handle for procedures; however, phylogenetic distance may affect the biological compatibility between the donor cells and the recipient testes. In this study, we evaluated the compatibility of lion (*Panthera leo*) SSCs and the domestic cat (*Felis catus*) testicular environment by comparing the phenotypic characteristics of their spermatogonial cells. Lion testes were collected from a male post-mortem, and a ~1 cm³ biopsy from each testis was fixed for paraffin embedding and sectioning. Tissue was reacted with antibodies for the surface markers KIT, GFRA1, and GPR125 commonly used in isolating SSCs in other mammalian species as well as pluripotent surface markers SSEA-1, SSEA-4, TRA-1-60, and TRA-1-81. Similar to domestic cat spermatogonia, lion spermatogonia expressed pluripotent markers SSEA-4, TRA-1-60, and TRA-1-81; however, the common SSC surface marker GFRA1 was also detected in lion spermatogonia whereas expression was observed throughout the seminiferous tubule in the domestic cat. These results provide preliminary evidence that lion and domestic cat SSCs may have similar biological characteristics, an indication that the domestic cat may be a suitable recipient for transplantation of lion SSCs.

Introduction

After the death of a male, a time period of only a few hours exists during which collection of any viable sperm is possible [115]. The amount of sperm is limited and, therefore, its utility is restricted. However, spermatogonia stem cells (SSCs), or sperm cell precursors, can be isolated from the testis and expanded *in vitro* for techniques to further propagate the

genetic material of the male. One such technique is SSC transplantation, which entails the transplantation of isolated SSCs to the testes of another male, and has been successful in fish and several mammalian species [21, 24, 37].

Xenogenic transplantation, or cross species transplantation, of SSCs has revealed that the greater the phylogenetic distance between the two species, the less likely the success of spermatogenesis of donor cells [9, 33]. Several studies have investigated the use of domestic species as models for conservation of related endangered exotic species, such as the use of domestic cats as recipients for interspecies embryo transfer [11, 88]. Recently, ocelot (*Leopardus pardalis*) mixed germ cells have been reported to successfully colonize and progress through spermatogenesis after transplantation to domestic cat testes [13]. In Chapters 1 and 2, we described the characterization of domestic cat spermatogonial cell subpopulations and demonstrated the ability of cells within those subpopulations to colonize recipient testes [78, 79, 116]. The main objectives of this study were: 1) to characterize lion (*Panthera leo*) testis cells using cell surface markers evaluated previously in the domestic cat and 2) to determine if the domestic cat is a suitable recipient for lion SSCs. Similarities in SSC expression patterns between the two felid species suggest a possible biological compatibility for SSCT, which would be confirmed by differentiation of lion SSCs into sperm following transplantation.

Materials and Methods

Testis procurement and handling. Testes were recovered from a recently deceased lion residing at the Audubon Zoo and were transported to the laboratory at ambient temperature in HEPES buffered saline solution (Ultrasaline A Solution, Bio-Whittaker, Walkersville, MD, USA). Testes were rinsed in Hanks' Balanced Salt Solution (HBSS; Sigma-Aldrich Chemical Co., St. Louis, MO, USA), and ~1 cm³ biopsies were taken from each testis for fixation and germ cell isolation.

Testis fixation. One biopsy from each testis was fixed, embedded in paraffin, and cut in serial sections of 5-7 µm for immunohistochemistry (IHC). Briefly, each tissue was incubated overnight at room temperature (rt) in 5 mL modified Davidson's fixative (mDf; Electron Microscopy Sciences, Hatfield, PA, USA). After decanting mDf from the tissue, 5 mL fresh mDf were added. Then, after a 24 h total fixation period, biopsies were submersed together in 10

mL 50% ethanol at rt for 2 h and stored in 10 mL of 70% ethanol until processed and embedded.

Immunohistochemistry (IHC). Testis sections were stained for KIT, GFRA1, GPR125, SSEA-1, SSEA-4, TRA-1-60, and TRA-1-81 (Table 1.1). Tissue sections were deparaffinized with Xylenes and rehydrated in decreasing serial dilutions of ethanol. A heat-induced epitope retrieval step using sodium citrate buffer was performed in a pressure cooker for 3 min after rocking was initiated. For antigen detection, the EXPOSE Rabbit specific HRP/DAB detection IHC kit (Abcam #80437, Eugene, OR, USA) was used according to the manufacturer's protocol with minor modifications. Tissue sections were blocked with a protein solution provided in the kit for 10 min, washed, and incubated with primary antibodies diluted with the protein solution at rt for 1.5 h. After washing with buffer solution, tissue sections were incubated for 10 min with a rabbit anti-mouse secondary antibody, followed by a 15 min incubation with a Horseradish Peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody. Chromogenic detection was completed with DAB (3, 3'-diaminobenzidine) in substrate solution until brown staining became visible, with incubation times ranging from 20 sec to 5 min. Stained sections were counterstained with hematoxylin for 30 sec, dehydrated, and a coverslip was applied with Permount (Thermo Fisher Scientific). For nonspecific controls, the primary antibody was replaced with goat IgG serum diluted with the protein block. Staining was visualized with an Olympus BX60 microscope, and images were captured with an Olympus DP72 digital camera.

Mixed germ cell isolation. Seminiferous tubules from biopsies of each testis were minced and incubated for 30-45 min in HBSS supplemented with 25 µg/mL amphotericin-B, 10 µg/mL streptomycin, and 50 µg/mL gentamicin. A mixed testicular cell suspension was obtained using a two-step enzymatic digestion, as previously described [9], then filtered through 100 µm and 40 µm nylon mesh filters (BD Falcon, Tewksbury, MA, USA) to remove remaining tissue and larger testicular cells. To separate sperm and cell debris from the spermatogonia, the resulting suspension was gently placed on a three-layer Percoll density gradient. Briefly, Percoll was diluted to gradient concentrations of 35.0%, 27.5% and 20.0% with HBSS, and layered in a 15 mL tube from highest to lowest concentrated gradient (Fig 2.1). The cells were centrifuged at 725 x g for 25 min. The bands formed at the 27.5% and 35.0%

Percoll layers, containing spermatogonial cells as identified by morphology, were pooled and rinsed three times with Dulbecco's phosphate buffered saline (DPBS). For differential adhesion culture, cells were re-suspended in DMEM/F12 medium (Gibco, Frederick, MD, USA) supplemented with 15% Knockout Serum Replacement (Invitrogen/Life Technologies, Carlsbad, CA, USA), 5% FBS (Hyclone/GE Healthcare Life Sciences, Piscataway, NJ, USA), 1 mM L-glutamine, 0.1 mM β -mercaptoethanol, 1.25% non-essential amino acids, 12.5 μ g/mL amphotericin-B, 10 μ g/mL streptomycin, and 50 μ g/mL gentamicin, and cultured four days at 38°C in a humidified atmosphere of 5% CO₂ in air.

Cell membrane staining with PKH26 fluorescence linker. To identify transplanted lion cells in the recipient's testes, cell membranes were labeled before transplantation with PKH26 Red Fluorescent Cell Linker Mini Kit, following manufacturer's guidelines with minor modifications. After differential plating, unattached germ cells (1.5×10^6) were centrifuged in DPBS at 310 x g for 5 min, supernatant discarded, and resultant pellets were re-suspended in 500 μ L of Diluent C (250 μ L; PKH26 Fluorescent Cell Linker Kit) and a dye solution (250 μ L Diluent C + 1.0 μ L PKH26), mixed by gentle pipetting and incubated for 5 min at rt. To bind the excess dye, an equal volume of DMEM/F12 medium was added to the stained cells, and the mixture was cultured for 1 min, washed, and re-suspended in a final volume of 200 μ L of DMEM/F12. Stained cells were evaluated for PKH26 fluorescence using an Olympus IX71 microscope and images were taken with an Olympus DP71 digital camera.

Transplantation of mixed germ cells and SSCs into prepubertal male cat recipients. Mixed germ cells were transplanted into the external rete testis of an 8-month-old early pubescent male recipient. First, the testes were externalized and removed from the tunica vaginalis. Then, the head of the epididymis was carefully micro-dissected away from the testis to expose the external rete testis. Germ cells were suspended in 100 μ L DMEM/F12 + 4 μ L trypan blue (for visualization during the injection), loaded into a 1-mL syringe, and injected into the external rete using a 28G needle.

Survival and colonization of transplanted mixed germ cells. The recipient was castrated at 11.5 weeks post-SSCT, testes were rinsed with HBSS, and the tunica vaginalis was removed from each. Then, epididymides were processed to detect spermatozoa, and

seminiferous tubules were analyzed for colony formation and progression through spermatogenesis.

To determine if donor sperm were produced, the epididymides were carefully removed and placed in separate dishes with HBSS for sperm extraction. The blunt edge of a scalpel blade was pressed along the length of each epididymis from the caput to the cauda, followed by multiple incisions. Then, the suspension was incubated for 30 – 45 min to allow sperm remaining within the epididymis to migrate into the medium. The presence of sperm was observed by conventional light microscopy (Olympus IX71, inverted), and samples of sperm were analyzed under fluorescence microscopy (Olympus BX60) to visualize the PKH26 fluorescence.

To evaluate colonization in the seminiferous tubules, testes were bisected longitudinally, one half of the testis was enzymatically digested, and seminiferous tubule fragments were immediately evaluated for donor colonization. The other half was snap-frozen for cross-sections analysis. For enzymatic digestion, the tunica albuginea was removed and seminiferous tubules were minced, transferred to a 15 mL conical tube containing 4 mL Collagenase IV (1 mg/mL; Gibco, Frederick, MD, USA), and incubated in a 38°C water bath for 30 min or until dissociation was complete. Dissociated seminiferous tubules were analyzed under fluorescence microscopy (Olympus IX71), and digital photographs were captured (Olympus DP71).

To snap-freeze, hemi-testis were placed in a tissue mold (VWR, Radnor, PA, USA) filled with OCT (VWR, Radnor, PA, USA), then submersed in a metal beaker containing a slush of isopentane (VWR, Radnor, PA, USA) chilled by liquid nitrogen. Frozen samples were stored at -80°C or on dry ice before cryosectioning. Slide cryosections were thawed at room temperature and the presence of red PKH26 fluorescence was observed microscopically (Olympus BX60). Then, tissue sections were stained with DAPI for 1 min, followed by gentle rinsing with DPBS, and re-examination and digital images were taken (Olympus DP72).

Results

Surface marker expression in lion testis. Cells stained positive for SSC surface marker GFRA1 (Fig 4.1) and pluripotent markers SSEA-4, TRA-1-60, and TRA-1-81 were restricted to the basal compartment of the seminiferous tubules (Fig 4.2). GFRA1 and SSEA-4 were detected in only a small number of single cells, whereas TRA-1-60 and TRA-1-81 were observed in pairs and chains of spermatogonia. Lion testis was negative for differentiation marker KIT, SSC surface marker GPR125 (Fig 4.1), and pluripotent marker SSEA-1 (Fig 4.2).

Figure 4.1. Immunohistochemical comparison of common SSC surface marker and differentiation marker expression in lion and domestic cat testis. Adult lion testis tissue was reacted with antibodies for GFRA1, GPR125, and KIT and compared to previously stained cat testis tissue. Brown staining indicates positive cells.

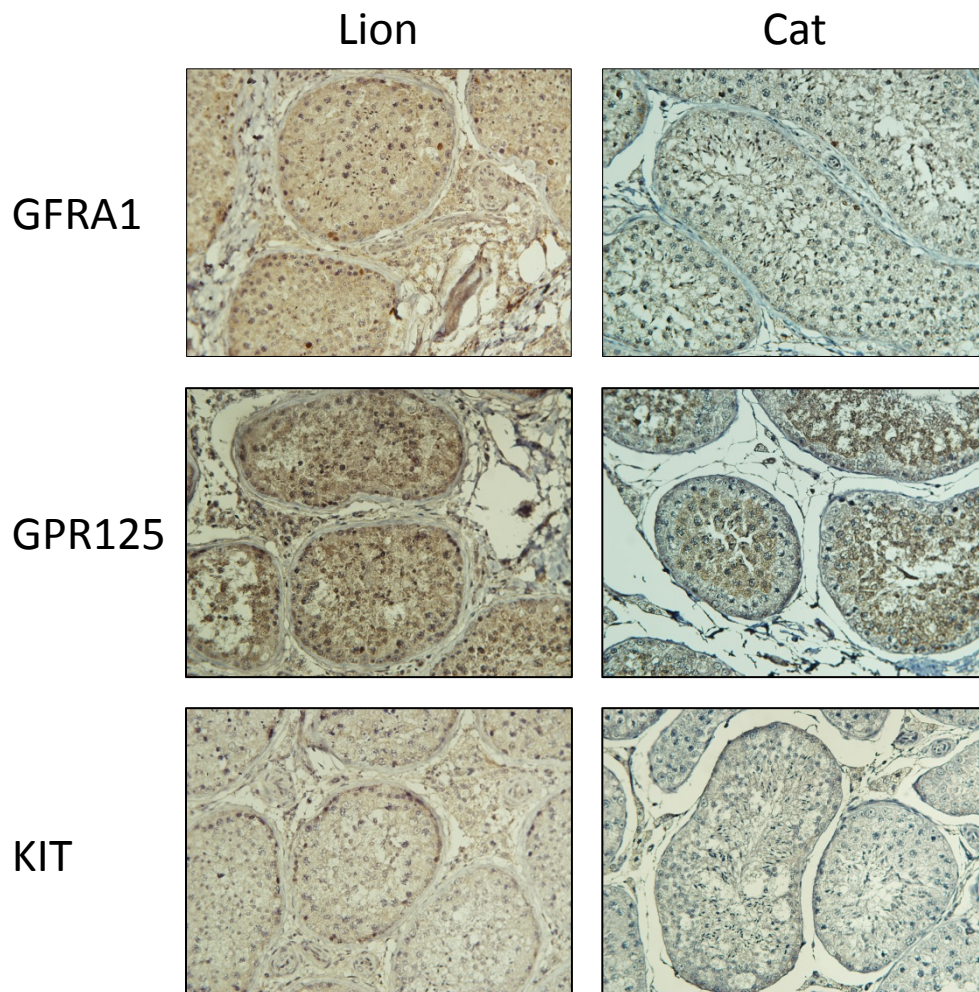
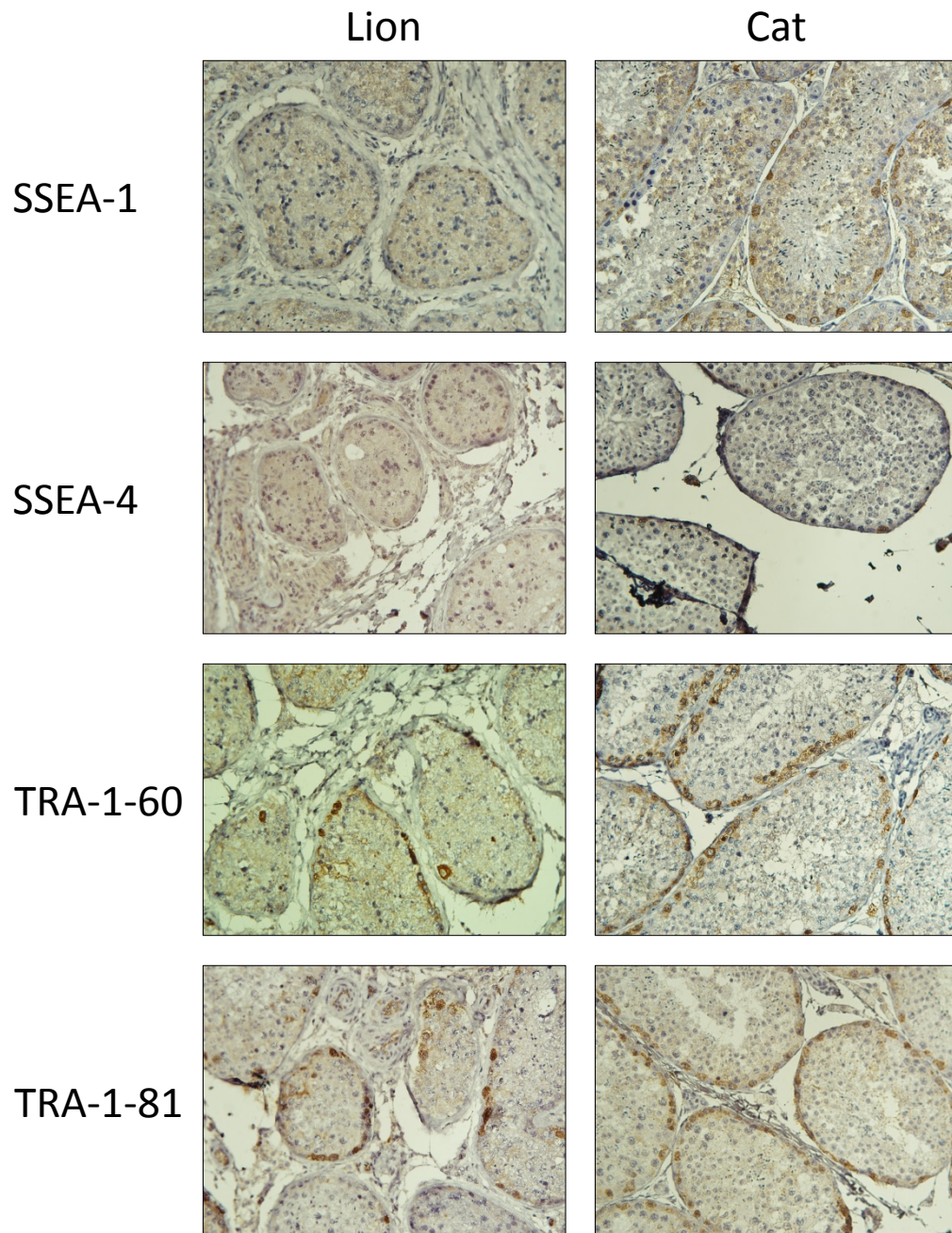
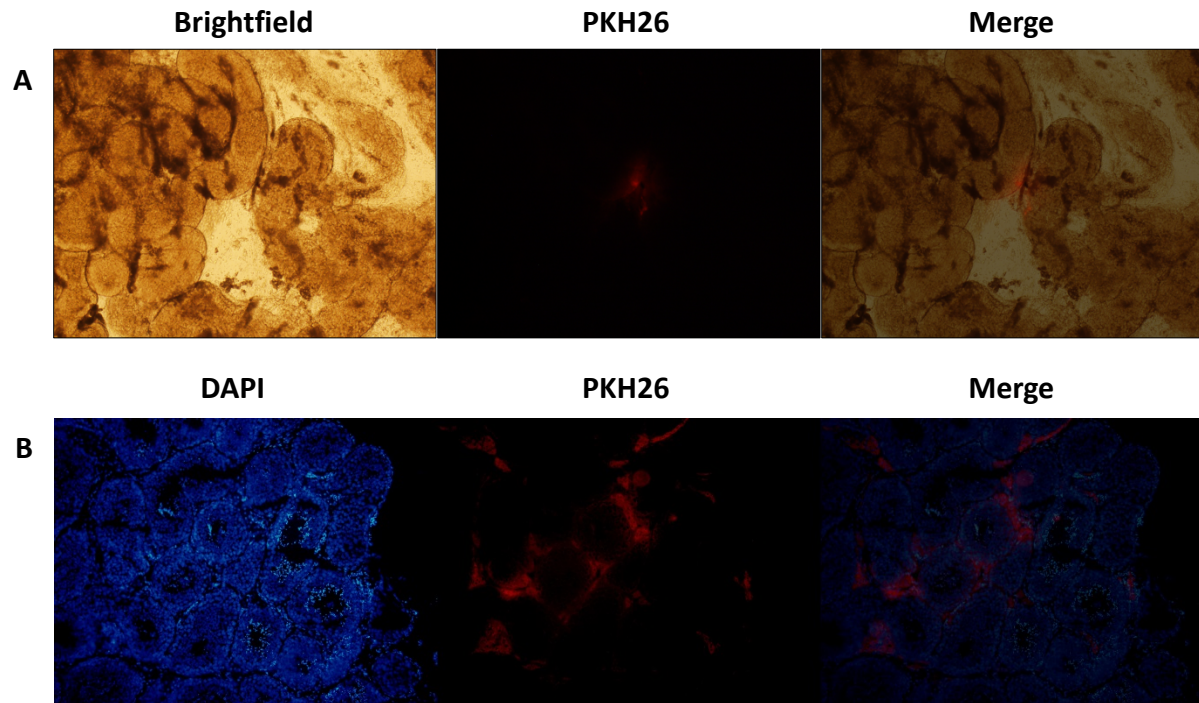


Figure 4.2. Immunohistochemical comparison of pluripotent surface marker expression in the lion and domestic cat testis. Adult lion testis tissue was reacted with antibodies for SSEA-1, SSEA-4, TRA-1-60, and TRA-1-81 and compared to previously stained cat testis tissue. Brown staining indicates positive cells.



Lion SSC colonization in cat testis. A low number of colonies was detected in the digested seminiferous tubule fragments of one testis (Fig 4.3 A), and colonization was confirmed in cross-sections of snap-frozen tissue, although some fluorescence appeared to be interstitial (Fig 4.3 B). Even though sperm was observed in the epididymal tissue, PKH26 fluorescence in sperm was not detected.

Figure 4.3. Donor cell colonies labeled with PKH26 red fluorescent membrane dye were detected in enzymatically digested tubules (A) and cross-sections (B) of the testes of some recipient cats. Nuclei in cross-sections were stained with DAPI.



Discussion

The efficacy of xenogenic SSCT is found to be heavily influenced by phylogenetic distance and biological dissimilarities between the species [33, 34]. For example, both rat and hamster SSCs were able to colonize mouse testes, but hamster germ cells developed abnormally while rat SSCs were reported to progress through spermatogenesis and result in normal sperm [36, 117]. We sought to determine if the domestic cat may be a viable recipient for lion SSCs by first comparing expression patterns of surface markers between the two species.

Similarities in expression patterns of pluripotent surface markers SSEA-4, TRA-1-60, and TRA-1-81 in lion and domestic cat cells were detected. In both species, SSEA-4 expression was restricted to single spermatogonia, while the other markers were found in pairs and chains of spermatogonia. However, pluripotent marker SSEA-1, although expressed in domestic cat spermatogonia, was not detected in lion testis. Other differences included GFRA1 expression localized to spermatogonia and negative expression of GPR125 in the lion as compared to non-specific expression for both surface markers in the domestic cat. Differentiation marker KIT was negative in testis tissue from both species.

Although donor cell colonization was observed in at least one of the two recipient testes, and sperm was present in the epididymis, donor-derived sperm were not identified. Previously, we reported colonization of domestic cat SSC colonization after syngenic transplantation to sexually immature recipient testes; however, sperm derived from donor cat cells were not observed [116]. It is not clear why donor sperm were not detected. It is possible that recipient endogenous germ cells at the time of transplantations competed with donor cells for a niche, especially in this study's eight-month-old early pubescent recipient with full spermatogenesis likely occurring, and the lack of donor sperm observed could be due to low, undetectable numbers of donor sperm. Isolating cells from the whole lion testis or larger biopsies would provide a greater number of mixed germ cells for transplantation, which may result in an increased number of donor colonies to produce detectable numbers of donor sperm. Furthermore, donor sperm production in recipient testes may increase with time, and extending the time between transplantation and sperm collection may also result in detectable numbers of donor sperm.

In summary, the lion and domestic cat share similarities in spermatogonial cell surface marker expression, and the presence of SSEA-4 in a low number of spermatogonia in both the lion and the domestic cat indicates SSEA-4 may be a potential surface marker for SSCs in other felids if expression is conserved. Although we did not detect sperm derived from lion SSCs after transplantation to domestic cat testes, it was reported that ocelot SSCs were able to differentiate and produce sperm in the domestic cat testicular environment [13]. Therefore, it

is necessary to repeat xenogenic lion SSCT and adjust the protocol as needed to confirm that the microenvironment of the domestic cat is compatible for lion SSC spermatogenesis.

GENERAL CONCLUSIONS

Isolation of spermatogonial stem cells (SSCs) is a technique for recovering genetic material which may be used for propagating valuable males of endangered species, especially when sperm collection is not possible. Although SSCs comprise <1% of the germ cells in the testis [27, 28], they concomitantly produce undifferentiated daughter cells and spermatogonia fated to differentiate into sperm throughout the life of the male. Transplantation of donor SSCs to the testes of an adequate recipient can result in *in vivo* donor sperm production, and it has been suggested that enrichment of SSCs through one or more methods, including differential plating and cell sorting using surface markers, may enhance colony formation efficiency.

Isolation of SSCs. The results presented in this dissertation revealed the presence of SSC subpopulations in the domestic cat and provided phenotypic and molecular characteristics for their identification. The low number of cells expressing pluripotent markers SSEA-1 ($15.4 \pm 7.1\%$), SSEA-4 ($5.9 \pm 2.9\%$), TRA-1-60 ($18.0 \pm 4.8\%$), and TRA-1-81 ($16.3 \pm 4.5\%$) compared to the more highly expressed common SSC surface markers GFRA1 ($44.7 \pm 2.5\%$) and GPR125 ($50.0 \pm 3.4\%$), and the differentiation marker KIT ($59.2 \pm 5.3\%$), suggested that the pluripotent markers are more specific to spermatogonia in the domestic cat. Detection of the pluripotent markers in prepubertal testis tissue and localization along the basement membrane of the seminiferous tubules in adult testis tissue confirmed that expression of SSEA-1, SSEA-4, TRA-1-60, and TRA-1-81 was restricted to spermatogonial cells. Furthermore, the expression of SSEA-4 in only single cell spermatogonia compared to pairs and chains of spermatogonia expressing SSEA-1, TRA-1-60, and TRA-1-81 indicated a change in phenotype with differentiation and that SSEA-4 may be more specific to SSCs.

Due to the lower percentage of cells positive for SSEA-1 or SSEA-4, populations of SSEA-1+ and SSEA-4+ cells were molecularly characterized for expression levels of pluripotent transcription factors *NANOG*, *POU5F1*, and *SOX2*, which are all expressed in cat embryonic cells [55]. Only *NANOG* was detected in SSEA-1+ cells, but all three pluripotent factors were expressed in SSEA-4+ cells. To further investigate the SSEA-4+ cell population, mixed germ cells were double stained and sorted for SSEA-4 and GFRA1, a receptor for a Sertoli cell-secreted

regulatory factor, which was detected in cat spermatogonia and differentiating germ cells. Three subpopulations were identified: 1) SSEA-4-, GFRA1+ ($29.3 \pm 17\%$), 2) SSEA-4+, GFRA1- ($0.7 \pm 0.5\%$), and 3) SSEA-4+, GFRA1+ ($3.7 \pm 2.8\%$). Interestingly, molecular analysis revealed that the SSEA-4+, GFRA1+ subpopulation expressed all three pluripotent transcription factors while expression was not seen in the SSEA-4-, GFRA1+ and SSEA-4+, GFRA1- subpopulations. These results suggest that SSEA-4+ SSCs also express GFRA1, and that the loss of either may signal differentiation.

Similar to my findings in the domestic cat, studies in human and non-human primates, including Rhesus macaque (*Macaca mulatta*) and the common marmoset (*Callithrix jacchus*), also reported expression of SSEA-4 in SSCs, and the percentage of SSEA-4+ cells ($\sim 6\%$) in the cat fell within the same range of SSEA-4+ cells in human ($\sim 13\%$) [20] and monkey SSCs ($\sim 2\%$) [74]. The definitive biological significance of SSEA-4+ SSCs in the cat remains unknown, but in humans, SSEA-4 proved an optimal marker for isolating SSCs [20, 32]. Furthermore, in both human and Rhesus monkey, portions of SSEA-4+ cells were found to co-express with cells positive for other surface markers such as ITGA6 [20, 32, 74], supporting other studies that found subpopulations of SSCs may exist that include a cohort of actively dividing stem cells and another of “reserve” stem cells that mostly remain latent [18, 118, 119]. The possibility of different roles of SSC subpopulations may explain the expression patterns of the pluripotent surface markers in single versus paired spermatogonia and the differing levels in expression of pluripotent transcription factors seen in the domestic cat.

Transplantation of SSCs (SSCT). Although successful SSCT was performed previously in domestic cats with the donor mixed germ cells from the cat and ocelot [13], I sought to evaluate: 1) if SSC-enriched SSEA-1+ or SSEA-4+ populations improve donor cell colonization and 2) if endogenous germ cell depletion using chemicals or irradiation in domestic cat recipients can be avoided by employing sexually immature males. Testicular weights of recipients at 10-12 weeks post-SSCT, ranging from 0.8 g to 1.25 g, were considered normal for seven- to nine-month-old male cats [14] and indicated spermatogenic activity. Although spermatozoa were present in the epididymides of 10 recipients (83%), PKH26 fluorescence in sperm was not observed. However, donor cell colonies localized to small areas along the basal

membrane of the seminiferous tubules were detected in five recipients (5/12; 42%): two receiving SSEA-4+ germ cells (2/4; 50%), one receiving SSEA-1+ germ cells (1/4; 25%), and two receiving mixed (unsorted) germ cells (2/4; 50%). Therefore, the SSEA-1+ and SSEA-4+ populations were capable of colonizing the seminiferous tubules of prepubertal cats after transplantation, confirming the presence of SSCs within those populations, but no advantage was found in colonization ability of the populations enriched for SSCs compared to each other or to mixed germ cell (unsorted) populations.

Although depleting endogenous germ cells in the prepubertal recipients was not necessary for successful colonization of donor cells, SSCT using prepubertal recipients with endogenous germ cell depletion is necessary for comparison to determine the efficiency of colonization in the untreated recipients. Previous studies indicated that the microenvironment of immature testes enhanced SSC colonization, allowing for the formation of more colonies than in the adult testes [17, 30]. The data suggested that glial cell line-derived neurotrophic factor (GDNF), which is important to SSC self-renewal, is secreted at higher levels by the immature testis, and the level of GDNF varies within the testis by the stage of seminiferous epithelium. Since the recipients in this study were just beginning spermatogenesis, the colonization of donor cells may have been restricted to only the segments of seminiferous tubule in which GDNF levels were high and a niche was available, accounting for the low number of donor colonies observed. It is possible that donor sperm were produced, but may have been too low in number to be detected; therefore, a more reliable method for detection of donor sperm in low numbers or over an extended period of time may be necessary.

Culture of SSCs. Expansion of domestic cat SSCs in an *in vitro* culture system would allow for a better understanding of the SSC biology to exploit their ability to self-renew or differentiate; however, defining culture conditions for cat SSC maintenance proved difficult. The effects of serum, a Sertoli cell feeder layer, B-27 supplement, and various combinations of growth factors GDNF, bFGF, EGF, and LIF on cat SSC proliferation were evaluated. I was able to maintain cat SSCs in the form of pairs, chains, or clusters *in vitro*, at least short-term, with stem cell medium containing several combinations of supplements, but only mixed germ cell populations formed compact, defined colonies.

Using mixed germ cells, I first determined that cells could survive up to two weeks without passaging either with or without the addition of fetal bovine serum (FBS); however, FBS promoted the immediate plating and rapid growth of somatic testicular cells. Few somatic cells plated without FBS present, and chains, grape-like clusters, and defined compact colonies of spermatogonia were observed. The effect of a mitotically inactivated Sertoli cell feeder layer, regardless of the presence of FBS, was difficult to evaluate. The Sertoli cells seemed sensitive to multiple passages and degenerated soon after inactivation and passaging. It was observed, however, that SSCs tend to start colonies on or near the plated somatic cells.

The proliferation effects of the B-27 supplement (without vitamin A) and growth factors GDNF, bFGF, EGF, and LIF were assessed in cultures of unsorted germ cells and sorted cells enriched in SSCs. After ~1 week, sorted cells became apoptotic in all culture conditions, possibly due to the lack of other supporting testicular cells. No noticeable effect on the culture of unsorted cells was observed under any of the culture conditions. All media evaluated contained GDNF, therefore, the most suitable medium tested for culturing cat SSCs was serum-free, containing 20% knockout serum replacement (KOSR), and supplemented with GDNF. However, further efforts in culturing and functional assays are needed to fully evaluate the effects of various factors and conditions, including the availability of somatic testicular cells, on sorted cells.

Xenogenic transplantation of SSCs. I was able to begin translating my findings in the domestic cat to another felid, the lion (*Panthera leo*). Surface markers tested in the cat were evaluated in tissue sections obtained from the testes of a recently deceased lion, and similarities in the expression patterns of the pluripotent markers SSEA-4, TRA-1-60, and TRA-1-81 were found, with SSEA-4 expression restricted to single spermatogonia and TRA-1-60 and TRA-1-81 expression detected in pairs and chains of spermatogonia. KIT was not detected in the testes of either species. Differences in expression of SSEA-1 and GPR125 between species were observed, with SSEA-1 undetected and GPR125 localized only in spermatogonia in the lion. Xenogenic transplantation of lion mixed germ cells resulted in colonization of donor cells, although sperm were not detected.

Final conclusions. These significant results not only indicate that SSEA-4 may be an optimal surface marker for felid SSC enrichment, but also reveal the presence of different domestic cat SSC subpopulations positive for SSEA-1 and SSEA-4 and the molecular characteristics of each. Furthermore, this study also shows that some of these characteristics are shared with the distantly related lion, an indication that the cat may be a viable recipient in xenogenic transplantation of other felid SSCs. Domestic cat testes have already been reported to be a biologically compatible environment for the colonization and differentiation of ocelot spermatogonia [13]. Although the ocelot lineage diverged 8 mya, more recently than the lion's *Panthera* lineage, approximately half of the other felid species are phylogenetically closer to the domestic cat than the ocelot [120-122], implying a likelihood that these species may also be compatible for xenogenic SSCT with the domestic cat as a recipient.

Therefore, xenogenic transplantation of exotic felid SSCs to the domestic cat may be an effective method in the propagation of genetically important males using a more manageable recipient for later collection of valuable donor sperm. Enrichment of feline SSCs through cell sorting with spermatogonia-specific surface markers and expansion in culture may offer several benefits including more efficient colonization, a continuous supply of valuable germ cells, and a better understanding of feline germ cell biology in the male. This is the first report of SSCT resulting in colonization using purified SSCs from FACS in the domestic cat. However, further work is needed to elucidate the factors and other conditions required for *in vitro* proliferation and to optimize the transplantation protocol to increase colonization efficiency and promote donor cell spermatogenesis.

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Appendix A. Approval from the Institutional Animal Care and Use Committee (IACUC).



Audubon Center for Research
of Endangered Species
A Facility of Audubon Nature Institute

Institutional Animal Care
and
Use Committee

June 20, 2012

Principal Investigator: Martha Gomez, DVM, PhD

RE: *IACUC Protocol No. 2012-04*
Entitled: "Transplantation of felid spermatogonial stem cells"

Dear Dr. Gomez:

The protocol proposal (referenced above) was reviewed and accepted by the Institutional Animal Care and Use Committee on June 18, 2012.

The animals' welfare, health and safety are priority. If the protocol causes any stress to the animals, the protocol results may be affected.

Sincerely,



Gwendolyn Alleman
Chair, IACUC

Appendix B. Approval letters from co-authors.



Health Sciences Center

NEW ORLEANS

School of Medicine
Department of Microbiology, Immunology and Parasitology

School of Medicine
School of Dentistry
School of Nursing
School of Allied Health Professions
School of Graduate Studies
School of Public Health

March 23, 2015

Dr. Kenneth Sewell, Executive Director
Graduate School
University of New Orleans
1004A Administration Annex
2000 Lakeshore Drive
New Orleans, LA

Re: Co-investigator/author Permission of Dissertational Inclusion of Manuscripts

Dear Dr. Sewell,

I grant permission for Robin Henry Powell to use in her doctoral dissertation any materials in the following manuscripts and other related works of which she is the principal researcher/author and I am the co-investigator/author:

Robin H. Powell, Jason Galiguis, Monica N. Biancardi, C. Earle Pope, Stanley P. Leibo, **Guoshun Wang**, Martha C. Gómez. "Phenotypic and Molecular Characterization of Domestic Cat Spermatogonial Stem Cells".

Robin H. Powell, Jason Galiguis, Qian Qin, Monica N. Biancardi, C. Earle Pope, Stanley P. Leibo, **Guoshun Wang**, Martha C. Gómez. "Successful Colonization of SSEA-1+ and SSEA-4+ Domestic Cat Spermatogonial Subpopulations after Transplantation into Pre-pubertal Males without Depletion of Endogenous Germ Cells".

Given any questions regarding this arrangement, please do not hesitate to contact me.

Sincerely,

A handwritten signature in blue ink, appearing to read "Guoshun Wang".

Guoshun Wang, DVM, PHD
CSRB 642, 533 Bolivar Street
New Orleans, LA 70112
Ofc#: 504-568-7908
E-mail: gwang@lsuhsc.edu

Dr. Kenneth Sewell, Executive Director
Graduate School
University of New Orleans
1004A Administration Annex
2000 Lakeshore Drive
New Orleans, LA

March 21, 2015

Dear Dr. Sewell,

I grant permission for Robin Henry Powell to use in her doctoral dissertation any material in which she is the principal researcher/author, including the following works currently being prepared for submission for publication of which I am a co-author:

Robin H. Powell, Jason Galiguis, Monica N. Biancardi, C. Earle Pope, Stanley P. Leibo, Guoshun Wang, Martha C. Gómez. "Phenotypic and Molecular Characterization of Domestic Cat Spermatogonial Stem Cells".

Robin H. Powell, Jason Galiguis, Qian Qin, Monica N. Biancardi, C. Earle Pope, Stanley P. Leibo, Guoshun Wang, Martha C. Gómez. "Successful Colonization of SSEA-1+ and SSEA-4+ Domestic Cat Spermatogonial Subpopulations after Transplantation into Pre-pubertal Males without Depletion of Endogenous Germ Cells".

Sincerely,

A handwritten signature in black ink, appearing to read "Jason Galiguis", with a stylized flourish at the end.

Jason Galiguis

Dr. Kenneth Sewell, Executive Director
Graduate School
University of New Orleans
1004A Administration Annex
2000 Lakeshore Drive
New Orleans, LA

March 23, 2015

Dear Dr. Sewell,

I grant permission for Robin Henry Powell to use in her doctoral dissertation any material in which she is the principal researcher/author, including the following work currently being prepared for submission for publication of which I am a co-author:

Robin H. Powell, Jason Galiguis, Qian Qin, Monica N. Biancardi, C. Earle Pope, Stanley P. Leibo, Guoshun Wang, Martha C. Gómez. "Successful Colonization of SSEA-1+ and SSEA-4+ Domestic Cat Spermatogonial Subpopulations after Transplantation into Prepubertal Males without Depletion of Endogenous Germ Cells".

Yours truly,

Qian Qin

VITA

Robin Henry Powell was born in San Francisco, California, but raised near New Orleans, Louisiana. She studied marine biology at Texas A & M University at Galveston for two and a half years before transferring to Louisiana State University where she completed her Bachelor's degree in biological sciences in 2002. While at LSU, she also took an interest in animal sciences and enrolled in several extra courses focusing specifically in animal reproduction. After graduation, she became involved in research investigating the virulence factors of *Helicobacter pylori* in gastric cancer patients at the Louisiana State University Health Sciences Center. On August 29, 2005, Hurricane Katrina prompted her to relocate to Houston, TX where she was accepted into a PhD program studying human and molecular genetics at the Graduate School of Biomedical Sciences at the University of Texas Health Sciences Center at Houston. In 2007, she transferred to the University of New Orleans in 2009 where she pursued a PhD in conservation biology with a focus in reproductive biology. Her research focused on manipulating spermatogonial stem cells in the domestic cat as a model for exotic felids. Under the supervision of Stanley P. Leibo, PhD and Martha Gomez, PhD, DVM, she conducted her dissertation research at the Audubon Center for Research of Endangered Species.